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(71) Applicant: **F. HOFFMANN-LA ROCHE AG**
Postfach 3255
CH-4002 Basel(CH)

(72) Inventor: **Fiers, Walter**
3 Beukendreef
B-9070 Destelbergen(BE)
Inventor: **Tavernier, Jan**
2 Bottelweg
B-9860 Balegem(BE)
Inventor: **Van Ostade, Xaveer**
46 Kipdorpvest
B-2000 Antwerp(BE)

(74) Representative: **Mezger, Wolfgang, Dr. et al**
Grenzacherstrasse 124 Postfach 3255
CH-4002 Basel(CH)

(54) TNF-Muteins.

(57) It is an object of the present invention to provide a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor, DNA sequences coding for such muteins, vectors comprising such DNA sequences, host cells transformed with such vectors and a process for the production of such muteins employing such transformed host cells and pharmaceutical compositions containing such muteins and their use for the treatment of illnesses, e.g. cancer.

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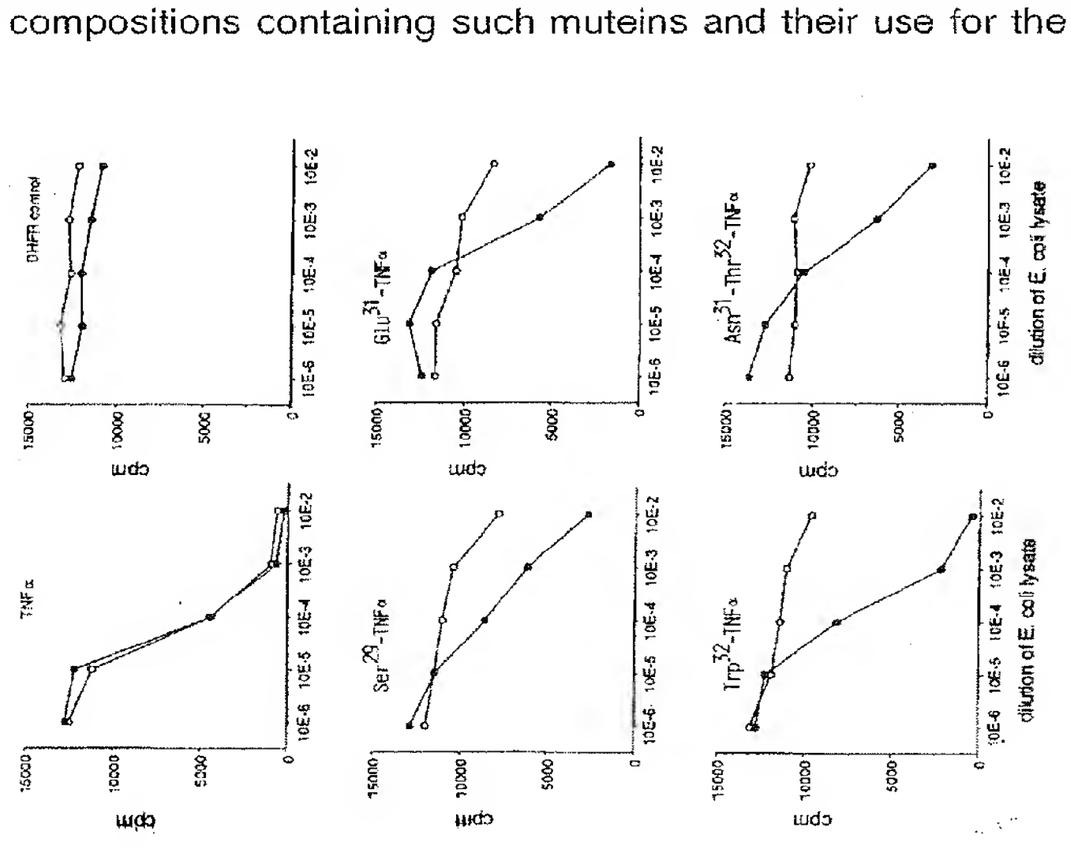

Fig. 6

Tumor Necrosis Factor, or more specifically Tumor Necrosis Factor-alpha, is a cytokine, primarily produced by stimulated macrophages, that exhibits not only a striking cytotoxicity against various tumour cells [Carswell et al., Procd. Nat. Acad. Sci., U.S.A. 72, 3666-3670, (1975)] but also plays a multiple role as a mediator of inflammation and the immune response [for an overview see Beutler and Cerami, Ann. Rev. Immunol. 7, 625-655 (1989); Bonavista and Granger (eds.) "Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy, Karger, Basel (1990)]. The primary structure of human Tumor Necrosis Factor-alpha (hTNF-α) has been deduced from the nucleotide sequence of a cDNA which has been cloned and expressed in E. coli [Pennica et al., Nature 312, 724-729 (1984); Marmenout et al., Europ. J. Biochem. 152, 515-522 (1985); Wang et al., Science 228, 149-154 (1985); Shirai et al., Nature 313, 803-806 (1985)]. A striking homology in amino acid sequence (30%) was found between hTNF-α and human Lymphotoxin, often referred to as human Tumor Necrosis Factor-beta (hTNF-β), a cytokine produced by a subset of lymphocytes [Gray et al., Nature 312, 721-724 (1984); Fiers et al., Cold Spring Harbour Symp. 51, 587-595 (1986)].

hTNF-α with modified amino acid sequences, so called TNF-α-muteins, have also been described in the art [for example see Yamagishi et al., Protein Engineering 3, 713-719, (1990) or by Fiers in "Tumor Necrosis Factors: Structure, Function and Mechanism of Action", Aggarwal and Vilcek (eds.), Marcel Dekker, Inc., New York, in press, or by Fiers et al. in Bonavista and Granger, pp. 77-81 (s.a.)]. In addition TNF-α-muteins have also been the object of several patent applications, e.g. International Patent Applications Publ. Nos. WO 86/02381, WO 86/04606, WO 88/06625 and European Patent Applications Publ. Nos. 155,549; 158,286; 168,214; 251,037 and 340,333, and Deutsche Offenlegungsschrift Nr. 3843534.

Muteins of Lymphotoxin have also been disclosed in the art, e.g. in European Patent Applications Publ. Nos. 250,000; 314,094 and 336,383.

The biological effects of TNF are mediated via specific receptors, namely a receptor with an apparent molecular weight of 55 kD on sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) (p55-TNF-R) and a receptor with an apparent molecular weight of 75 kD on SDS-PAGE (p75-TNF-R). Both forms of TNF-receptors have been cloned, namely p55-TNF-R by Loetscher et al. [Cell 61, 351-359, (1990)] and p75-TNF-R by Dembic et al. [Cytokine 2, 53-58, (1990)] (for both receptors see also European Patent Application No. 90116707.2) and it was found more recently that both receptors bind not only TNF-α but also TNF-β with high affinity [Schönfeld et al., J. Biol. Chem. 266, 3863-3869 (1991)].

Object of the present invention is a mutein or a pharmaceutically acceptable salt thereof on the basis of the amino acid sequence of human Tumor Necrosis Factor which sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and the human p55-Tumor-Necrosis-Factor-Receptor.

A preferred embodiment of the present invention is a mutein as defined above on the basis of the amino acid sequence of TNF-α as disclosed by Pennica et al. [s.a.], namely:

```
1                                                  10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

                  20                                                  30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

                                                   40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP
```

```
                    50                                                  60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

                                                    70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

                    80                                                  90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

                                                   100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

                   110                                                 120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

                                                   130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU

                   140                                                 150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL

                   157
TYR PHE GLY ILE ILE ALA LEU
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or as disclosed by Marmenout et al. (s.a.) or Wang et al. (s.a.) or Shirai et al. or more specifically as coded for by the nucleotide sequence of the insert of the plasmid pDS56/RBSII,Sph1-TNFα (see Figures 3a and 3b and Example I) coding for mature TNF-α.

A specifically preferred embodiment of the present invention is a mutein as defined above wherein the TNF-α amino acid sequence is changed by substitution of one or more amino acids, preferably one or two by other amino acids, preferably naturally occuring amino acids.

More specifically preferred embodiments of the present invention are muteins as defined above wherein the TNF-α amino acid sequence is substituted at position 29 and/or 32 or position 31 and 32 or position 31 or position 29 and 31 whereby substitutions at position 29 and/or 32 or position 31 and 32 or position 31 are preferred (referring to a TNF-α amino acid sequence with 157 amino acids) by other amino acids, preferably naturally occuring amino acids. Any amino acid, preferably any naturally occuring one, can be used at this position which leads to a TNF-mutein showing a significant difference between its binding affinity to the human p75-TNF-R and the human p55-TNF-R, whereby for substitutions at position 29 serine, glycine or tyrosine are preferred whereby serine is especially preferred, e.g. in case of a single position mutein at position 29 (Ser^{29}-TNFα). For substitutions at position 31 glutamic acid, e.g. Glu^{31}-TNFα, or asparagine are preferred. For substitutions at position 32 tyrosine, e.g. Tyr^{32}-TNFα or tryptophan, e.g. Trp^{32}-TNFα are preferred, whereby the latter one is specifically preferred. Especially preferred substitutions in case of a double position mutein at positions 29 and 32 are Ser^{29}-Trp^{32}-TNFα and at position 31 and 32 are Asn^{31}-Thr^{32}-TNFα. It is understood that the muteins of the present invention can also be prepared by methods known in the art of chemical peptide and protein synthesis, e.g. by partial or total liquid or solid phase synthesis as described e.g. by Gross and Meyenhofer in "The Peptides" Vols. 1-9, Academic Press, Inc., Harcourt Brace Jovanovich, Publs., San Diego (1979-1987) or by Fields and Nobel, Int. J. Pept. Prot. Res 35, 161-214 (1990).

Analogs obtained by deletion, substitution and/or addition of one or several amino acids from or to the muteins as defined in the previous paragraph whereby position 29 and/or 32 or position 31 or position 31 and 32 in the mutein is/are not changed and which analogs still show a significant difference between its binding affinity to the human p75-TNF-R and the human p55-TNF-R are also an object of the present invention. With respect to such substitution analogs amino acid substitutions in proteins which do not generally alter the activity are known in the state of the art and are described, for example, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr. Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse (the three letter abbreviations are used for amino acids and are standard and known in the art).

Substitution, addition and/or deletion analogs can be produced by methods known in the art and described e.g. in Sambrook et al. [Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbour Laboratory Press, USA (1989)] or in the following paragraphs. Whether such an analog still shows the significant difference between its binding affinity to the p75-TNF-R and the p55-TNF-R can be determined as described in the following and e.g. more specifically in Examples IIa) and b) or Example VIII. Furthermore salts of such muteins and analogs are also an object of the present invention. Such salts can be produced by methods known in the art.

It is furthermore an object of the present invention to provide a mutein as described above for the treatment of illnesses, e.g. cancer.

It is well known in the art that on the basis of its biological activities (s.a.) TNF-α can be a valuable compound for the treatment of various disorders. For example TNF-α, alone or in combination with interferon, can be an effective antitumor agent [Brouckaert et al., Int. J. Cancer 38, 763-769 (1986)]. However, its systemic toxicity is a major limitation to its wider therapeutic use [Taguchi T. and Sohmura Y., Biotherapy 3, 177-186 (1991)].

The discovery of two TNF-receptors with (putatively) distinct functional roles should allow to dissect in a given disease state the benefical and unwanted biological responses to TNF. There is circumstantial evidence supporting the feasibility of this approach. It has been shown for example [Brouckaert et al., Agents and Actions 26, 196-197 (1989); Everaerdt, B. et al., Biochem. Biophys. Res. Comm. 163, 378-385 (1989)] that in mice murine TNF-α (mTNF-α) is up to 50-fold more toxic than human TNF-α (hTNF-α), although when tested in cell culture, both are equally active on sensitive cell lines.

It is believed that the strategy of dissecting beneficial and unwanted TNFα activities by using compounds specifically binding to one or the other TNF-receptor, such as the TNF-muteins of the present invention, can be used in general in other disease states where TNF plays a role.

DNA-sequences comprising a DNA-sequence coding for TNF-muteins as hereinbefore described are also an object of the present invention. Such DNA-sequences can be constructed starting from genomic- or cDNA-sequences coding for hTNF as disclosed in the art [s.a.] using known methods of in vitro mutagenesis [see e.g. Sambrook et al., 1989]. Such mutagenesis can be carried out ad-random in order to obtain a large number of mutants which can than be tested for their desired properties in appropriate assay systems or, in order to mutate defined positions in a given DNA-sequence, by so called site directed mutagenesis [see e.g. Sambrook et al., 1989, 15.51-15.113] or by mutagenesis using the polymerase chain reaction [see e.g. White et al., Trends in Genetics 5, 185-189 (1989)].

One chemical mutagen which is often used for mutagenesis ad-random is sodium bisulfite which converts a cytosin residue into an uracil residue and hence leads to a transition of "C" to "T" (standard abbreviations for nucleotides) [for the method see e.g. Shortle and Nathans, Procd. Nat. Acad. Sci. U.S.A. 75, 2170-2174 (1978) or Pine and Huang, Meth. Enzym. 154, 415-430 (1987)]. This mutagen acts solely on single stranded DNA whereas the expression of the mutated target DNA sequence is achieved with a double stranded plasmid vector. One possibility to avoid the necessity of recloning in mutagenesis and expression vectors is the use of so called "phasmids". These are vectors which, in addition to a plasmid origin of replication, carry also an origin of replication derived from a filamentous phage. Examples of such phasmids are the pMa- and pMc-phasmids as described by Stanssen et al. [Nucleic Acids Res. 17, 4441-4454, (1989)]. Using this expression system one can construct so called "gap-duplex"-structures [see also Kramer et al., Nucl. Acids. Res. 12, 9441-9456 (1984)] where only the TNF-coding sequence (s.a.) is in a single stranded configuration and therefore accessible for the specific chemical mutagen. "gap-duplexes" to be used in ad-random mutagenesis can be constructed as described for site-specific mutagenesis by Stanssen et al. [s.a.] with the exception that the (-)strand contains the same active antibiotic resistance gene as the (+)strand. By making use of different restriction sites in the DNA-sequence encoding hTNFα, variation of the width of the gap is possible. Examples of such restriction sites are the C1a1-Sal1 sites (470 nucleotides), BstX1-BstX1 sites (237 nucleotides) or Sty1-Sty1 sites (68 nucleotides). Such gap-duplex-constructs can then be treated with increasing concentrations (up to 4M) of bisulfite, followed by several dialysis steps, as described by Shortle and Nathans (s.a.). A suitable procaryotic host cell can then be transformed by such phasmid constructs according to methods known in the art and described e.g. by Sambrook et al. (s.a.). A suitable procaryotic host cell means in this context a host cell deficient in a specific repair function so that an uracil residue is maintained in the DNA during replication and which host cell is capable of expressing the corresponding mutated TNF. Such specific host strains are known in the art, for example for E. coli strains, e.g. E. coli BW 313 [Kunkel, T.A., Procd. Natl. Acad. Sci. USA 82, 488-492 (1985)]. The resulting clones can then be screened for those expressing a desired TNF-mutein by appropriate assay systems. For example each colony can be inoculated in a microtiterplate in a suitable medium containing the relevant antibiotic. The cells may be lysed by addition of lysozyme, followed by

sequential freeze-thaw cycles. After precipitation of nucleic acids and centrifugation, the supernatant of each colony can directly be used in appropriate assays as described, e.g., in Example IIa and IIb or Example VIII measuring binding to the p75-TNF-R and the p55-TNF-R on the surface of living cells or in purified form.

If desired, the specific sites of mutation can be determined, for example by restriction fragment analysis [see e.g. Sambrook et al. (s.a.)]. By determination of the DNA-sequence of such fragments the exact position of the mutation can be determined and if such mutation leads to an amino acid replacement the new amino acid can be derived from the determined DNA-sequence. DNA-sequencing can be performed according to methods known in the art, e.g. by using T7 polymerase on supercoiled DNA with a commercially available sequencing kit (Pharmacia, Uppsala, Sweden).

As already mentioned above, another possibility of mutating a given DNA-sequence is by "site directed mutagenesis". A widely used strategy for such kind of mutagenesis as originally outlined by Hutchinson and Edgell [J. Virol. 8, 181 (1971)] involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single stranded DNA-sequence wherein the mutation should be introduced [for review see Smith, Annual. Rev. Genet. 19, 423 (1985) and for improved methods see references 2-6 in Stanssen et al. (1989)].

One such preferred method is the one of Stanssen et al. (1989) using "gapped duplex DNA" as originally described by Kramer et al. (1984) [see above and Kramer and Fritz, Methods in Enzymology, (1987), Academic Press, Inc., USA] but using antibiotic resistance genes instead of M13 functional genes for selection of the mutation containing strand in addition with the phasmid-technology as also described by Stanssen et al. (1989) [s.a.]. An advantage of this method lies also in the capability of performing successive cycles of mutagenesis without the need to transfer the gene to a new mutagenesis vector: second round mutagenesis differs only in the selection to another antibiotic marker (Stranssen et al., s.a.). As a control site-specific back mutagenesis of the mutant to the wild-type TNF can be used. In addition, the use of an oligonucleotide, creating or destroying a restriction site in the TNF gene, allows to control the mutant not only by hybridization to the oligonucleotide used for site directed mutagenesis but also by the presence or absence of the restriction site. In order to create a set of TNF-muteins wherein at a defined position of their amino acid sequence the wild-type amino acid is replaced by any naturally occurring amino acid a set of oligonucleotides is used with all possible codons at the defined position.

As already mentioned above, another possibility of mutating a given DNA-sequence is the mutagenesis by using the polymerase chain reaction (PCR). The principles of this method are outlined e.g. by White et al. (1989), whereas improved methods are described e.g. in Innis et al. [PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990)].

PCR is an in vitro method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a template DNA. Thereby, PCR is based on the enzymatic amplification of the DNA fragment which is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. Since the primers are physically incorporated into the amplified product and mismatches between the 5' end of the primer and the template do not significantly affect the efficiency of the amplification, it is possible to alter the amplified sequence thereby introducing the desired mutation into the amplified DNA. By utilizing the thermostable Taq DNA polymerase isolated from the thermophilic bacteria Thermus aquaticus, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers.

Design and synthesis of oligonucleotides can be effected as known in the art and described e.g. in Sambrook et al. (1989) or in one of the references cited above with respect to site directed mutagenesis.

As soon as a DNA-sequence coding for a TNF-mutein of the present invention has been created, expression can be effected by the phasmid technology as described above or by use of any suitable pro-or eukaryotic expression system well known in the art [see e.g. Sambrook et al., s.a.].

Expression is effected preferably in prokaryotic cells, e.g., in E. coli, Bacillus subtilis and so on, whereby E. coli, specifically E. coli K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694], WK6 (Stranssens et al. s.a.) or E. coli SG13009

[Gottesman et al., J. Bacteriol. 148, 265-273 (1981)] are preferred. Expression of the muteins of the present invention can also be effected in lower or higher eukaryotic cells, like for example yeast cells (like Saccharomyces, Pichia etc.), filamentous fungi (like Aspergillus etc.) or cell lines (like chinese hamster ovary cell lines etc.), whereby expression in yeast cells is preferred [see Sreekrishna et al., Biochem. 28, 4117-4125, (1989); Hitzeman et al., Nature 293, 717-722 (1981); European Patent Application Publication No. 263 311]. Expression of the TNF-muteins of the present invention may occur in such systems either intracellularly, or, after suitable adaption of the gene, extracellularly (see Leemans et al., Gene 85, 99-108, 1989).

Suitable vectors used for expression in E. coli are mentioned e.g. by Sambrook et al. [s.a.] or by Fiers et al. in "Procd. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris, (Durand et al., eds.), pp. 680-697 (1988)] or and more specifically vectors of the pDS' family [Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987); Stüber et al., Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990)] like for example pDS56/RBSII,Sph1-TNFαSer29 or pDS56/RBSII,Sph1-TNFαTrp32 (see Example I) or pDS56/RBSII,Sph1-TNFαGlu31 or pDS56/RBSII,Sph1-TNFαAsn31Thr32 (see Example VII). The transformed E. coli strains M15 (pREP4;pDS56/RBSII,Sph1-TNFαGlu31) and M15 (pREP4;pDS56/RBSII,Sph1-TNFαAsn31Thr32) have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, BRD at September 8th, 1991 under accession numbers DSM 6714 and DSM 6715 respectively. Since with these specific pDS56/RBSII-plasmids due to their specific regulatable promoter/operator elements and ribosomal binding sites a high level of expression can be achieved, the plasmids can be maintained in E. coli cells only when the activity of the promoter/operator element is repressed by the binding of a lac repressor to the operator. The activity of the promoter can be restored at the desired cell density by addition of IPTG, which inactivates the repressor and clears the promoter. Since most of the E. coli strains do not provide enough repressor molecules to completely repress the function of the promoter sequences present in these high copy number plasmids, such E. coli strains, like E. coli M15 or SG13009, have to be transformed at first with a plasmid, like pREP 4, coding for the lac repressor, before being transformed with the specific pDS56/RBSII-plasmids of the invention which can then be stably maintained in the E. coli cells. Beside coding for the lac repressor, pREP4 contains also a region of the plasmid pACYC184 [Chang and Cohen, J. Bacteriol. 134, 1141-1156 (1978)], which contains all information required for replication and stable transmission to daughter cells [for additional information see also "System for high level production in E. coli and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure function analysis" by Stüber et al. in Immunological Methods, Vol. IV, pp 121-152, Lefkovits and Pernis (eds.), Academic Press, New York (1990).

Transformation of the host cells by vectors as described above may be carried out by any conventional procedure [see for example Sambrook et al. (s.a.)]. Where the host cell is a prokaryote, such as E. coli for example, competent cells which are capable of DNA uptake are prepared from cells harvested after exponential growth phase and subsequently treated according to the known $CaCl_2$-method. Transformation can also be performed after forming a protoplast of the host cell or by other methods known in the art and described, e.g., in Sambrook et al. (s.a.). Therefore a vector, especially for expression in a prokaryotic or lower eukaryotic host cell, comprising a DNA-sequence coding for a TNF-mutein as described above, and a host cell, especially a prokaryotic host cell, e.g. E. coli, or a lower eukaryotic host cell, transformed by such a vector are also an object of the present invention.

Usually, the host organisms which contain a desired expression vector are grown under conditions which are optimal for their growth. In case of a procaryotic host at the end of the exponential growth, when the increase in cell number per unit time decreases, the expression of the desired TNF-mutein is induced, i.e. the DNA coding for the desired TNF-mutein is transcribed and the transcribed mRNA is translated. The induction can be carried out by adding an inducer or a derepressor to the growth medium or by altering a physical parameter, e.g. a change in temperature. In the expression vectors used in the preferred embodiments of the present invention, the expression is controlled by the lac repressor. By adding isopropyl-β-D-thiogalactopyranoside (IPTG), the expression control sequence is derepressed and the synthesis of the desired TNF-mutein is thereby induced.

TNF-muteins of the present invention produced by transformed host cells as stated above can be recovered from the culture medium or after opening the cells and/or extraction by any appropriate method known in protein and peptide chemistry such as, for example, precipitation with ammonium sulfate, dialysis, ultrafiltration, gelfiltration or ion-exchange chromatography, gel electrophoresis, isoelectric focusing, affinity chromatography, like immunoaffinity chromatography, HPLC or the like. Specifically preferred methods are precipitation with ammonium sulfate and/or polyethylenimine, dialysis, affinity chromatography, e.g. on

phenyl-agarose, specifically phenyl-sepharose, or ion-exchange chromatography, specifically on a MONO-Q- and/or MONO-S-matrix (Pharmacia, Uppsala, Sweden) or more specifically are those as described by Tavernier et al. [J. Mol. Biol. 211, 493-501 (1990)] and those disclosed in Example I or Example III.

It is therefore also an object of the present invention to provide a process for the preparation of a compound as specified above which process comprises cultivating a transformed host cell as described above in a suitable medium and isolating a mutein from the culture supernatant or the host cell itself, and if desired converting said mutein into a pharmaceutically acceptable salt. The compounds whenever prepared according to such a process are also an object of the present invention.

The muteins of the present invention are characterized by showing a significant difference between its binding affinity to the human p75-TNF-R and the human p55-TNF-R. Such property can be determined by any assay known in the art measuring binding affinities. For example the binding of TNF itself and of the muteins of the present invention can be measured using cells in cell culture which express the two types of TNF-receptors to a different degree, like for example Hep-2 cells which exclusivly express the human p55-TNF-R and U937 or HL60 cells which express in addition also the human p75-TNF-R [see Brockhaus et al., Procd. Nat. Acad. Sci. U.S.A. 87, 3127-3131, (1990); Hohmann et al., J. Biol. Chem. 264, 14927-14934, (1989); Loetscher et al. (1990); Dembic et al. (1990)]. Of course binding affinities can also be determined directly by using purified native or recombinant p55-TNF-R and p75-TNF-R as specifically described in Example IIb, or by using the corresponding soluble analogs of such receptors.

The term significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor" refers in the context of the present invention to a difference in binding affinities to the two types of TNF-receptors which is with respect to the used assay system significant enough to say that a mutein of the present invention binds preferentially to one of the two TNF-receptors as compared to wild type TNF. More specifically this term means in the context of the assay-system of Example IIa) that a K_D-value of a specific TNF-mutein of the present invention is at least a factor of 10 or more, especially preferred at least a factor of 10^2, larger than for TNF-α itself determined by using U937 cells whereby its K_D-value determined by using Hep-2 cells for the same TNF-mutein is not larger than a factor of 2 as for TNF-α: itself [for specific data see Table I of Example IIa)-]. It is however understood that these specific K_D-values are given for illustration and should not be considered as limiting in any manner.

The muteins of the present invention can be characterized by their anti-tumour activity by methods known in the art and described e.g. in Example IV.

The muteins of the present invention may show but not necessarily considerably reduced cytotoxic activity in standard TNF-assays which are based on murine cell lines, such as L929 (see Table 1) or L-M cell lines.

TNF-muteins of the present invention can be used for the treatment of illnesses. e.g. cancer.

A further object of the present invention is a pharmaceutical composition and a process for its preparation which composition contains one or more compounds of the invention, if desired in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials. For this purpose, one or more compounds of the invention, where desired or required in combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure. After the invention has been described in general hereinbefore, the following Examples are intended to illustrate details of the invention, without thereby limiting it in any manner.

Examples

Example I

Preparation of Ser^{29}-TNFα and Trp^{32}-TNFα

Construction of a mutagenesis vector

From the human TNF expression plasmid pDS56/RBSII,Sph1-TNFα (see Figure 3a: The expression plasmid contain the regulatable promoter/operator element N25OPSN25OP29

( ),

the synthetic ribosomal binding site RBSII

(),

genes

()

for β-lactamase (bla), chloramphenicol acetyltransferase (cat), and transcriptional terminatos

()

t_o of phage lambda (t_o) and T1 of rrnB operon of E. coli (T1), and the replication region of plasmid pBR322 (repl.). The coding region under control of N25OPSN25OP29 and RBSII is indicated by an arrow; for complete nucleotide sequence of the plasmid see Figure 3b/1-3b/3 given by the one letter standard abreviations for nucleotides), an EcoR1-HindIII fragment was isolated, containing the ribosome binding site RBSII, the mature TNFα coding sequence and a 130 bp 3' non-translated sequence. This fragment was cloned into the EcoR1-HindIII opened pMac phasmids (Stanssens et al., s.a.), resulting in the constructions pMa/RBSII,Sph1-TNFα and pMc/RBSII,Sph1-TNFα.

Isolation of single-stranded (ss)DNA

The pMa/RBSII,Sph1-TNFα phasmid was transformed to E. coli WK6 (Stranssens et al., s.a.). One colony was picked up and cultured in 5 ml LB medium (Sambrook et al., 1989) + carbenicillin (50 µg/ml) at 37 ° C, overnight. 1 ml of this confluent culture was used to inoculate 200 ml LB + carbenicillin. When the absorbance (650 nm) reached a value of 0.1, the culture was infected with M13K07 helper phage (Stanssens et al., (1989) at a m.o.i. of about 20 and further incubated overnight at 37 ° C. Then, the cells were spun down (10 min, 10.000 rpm) and the supernatant was transferred into another tube. 50 ml PEG-solution (20% polyethylene glycol 6000; 2.5 M NaC1) was added and the mixture was kept on ice for one hour to precipitate the phages. After centrifugation (10 min; 8000 rpm), the supernatant was removed and the tube was dried on paper towels for 10 min. The phage pellet was resuspended in 6 ml TE buffer (10 mM Tris-HC1, 0.1 mM EDTA, pH8). A first extraction was performed with 6 ml TE-saturated phenol, followed by vortexing for 3 min. After centrifugation (3 min) in an Eppendorf centrifuge, the aqueous phase was transferred to a fresh tube and a second extraction was carried out with chloroform:isoamylalcohol (24:1), the same way as described. The single stranded DNA could be precipitated by adding 1/10 volume of 5M $NaC10_4$ and 1 volume of isopropanol (-20 ° C, 2 hours). This ssDNA was pelleted by centrifugation for 20 min in an Eppendorf centrifuge. The pellet was dried and dissolved in 500 µl TE buffer as a control, 5 µl of this mixture was run on an agarose gel, containing 1 µg/ml ethidium bromide. Usually, the ratio of pMa/RBSII,Sph1-TNFα ssDNA (=(+)strand) over helper phage ssDNA was between 2:1 and 20:1. The amount of total ssDNA was estimated to be at least 200 ng/µl.

Construction of a gap-duplex

From the phasmid pMc, the EcoR1-HindIII large fragment was isolated and used for hybridization to the pMa/RBSII, Sph1-TNFα(+)strand. In a typical experiment, 15 µl ssDNA (± 3 µg), 15 µl of the double stranded, linear fragment (± 1.5 µg), 10 ml hybridization buffer (1.5 M KC1; 100 mM Tris-HC1, pH 7.5) and 40 µl H_2O were mixed and incubated at 100 ° C for 4 min, 65 ° C for 8 min and room temperature for 15 min. An aliquot (10 ml) was electroforesed on an agarose gel containing ethidium bromide, to check the formation of gap duplex DNA and, if so, to estimate its quantity (this usually amounted to 50ng/10ml hybridization mixture). Annealing of the mutant oligonucleotide and fill-in of the gap duplex Oligonucleotides were synthesized containing the mutated codon and destroying or creating a restriction site in the TNF gene. The oligonucleotides 5'CCGGCGGTTGGACCACTGGAGC3' and 5'CATTGGCCCAGCGGTTCAG3' (mutated bases underlined) were used to create the Ser^{29} and Trp^{32} mutations respectively. After enzymatic phosphorylation, about 8 pmol was added to 40 ng of gap-duplex. H_2O was added to a final volume of 10 ml. This mixture was heated to 65 ° C for 5 min and allowed to cool to room temperature. Subsequently, 18

ml H_2O, 4 µl fill-in buffer 10 (625 mM KCl, 275 mMTris-HCl, 150 mM $MgCl_2$, 20 mM DTT pH 7.5), 2 µl ATP 1mM, 4 µl of the four dNTP's 1mM, 1 µl ligase and 1 ml Klenow polymerase were added and the mixture was incubated at room temperature for 45 min.

Transformation to E. coli WK6 mutS and E. coli WK6

We used 10 µl of the filled-in gap duplex DNA to transform (Sambrook et al., 1989) E. coli WK6 mutS (Stranssens et al., s.a.). From this mixture (1.2 ml), 100 ml was plated out on agar plates containing 25 µg/ml chloramphenicol to check transformation efficiency. The remainder was used to inoculate 20 ml LB + chloramphenicol and further grown overnight at 25 ° C. A small-scale plasmid DNA preparation [Birnboim, H.C. and Doly, J., Nucleic Acids Res., 7, 1513, (1979)] of this culture (not yet grown to confluency) resulted in a mixed phasmid population that could be transformed to E. coli WK6. Again, 100 µl transformation mixture was plated out on agar plates containing chloramphenicol.

Screening by colony hybridization

About 100 colonies, resulting from the transformation to E. coli WK6, were streaked on a nylon filter (PALL, Glen Cove, New York) and incubated overnight at 37 ° C. The filter was transferred (face up) to Whatmann 3MM papers which were soaked in 0.5 M NaOH (3 min). Neutralization was done by transfer to Whatmann 3MM sheets soaked in 1M Tris-HCl pH 7.4 (twice for 1 min) and 2XSSC (20xSSC = 3M NaC1; 0.3M Na citrate, pH7) (5 min). After drying, the filter was baked at 80 ° C between sheets of 3MM paper. Subsequently, the filter was prewetted in 6xSSC (5 min) and prehybridized at 67 ° C for 5 min in 10x Denhardt solution (2% (w/v) Fico11 (400,000 MV), 2% (w/v) Polyvinylpyrrolidone (44,000 MW), 2% (w/v) Bovine Serum Albumin), 6xSSC buffer and 0.2% SDS. After rinsing in 6xSSC buffer, the filter was placed in a Petri dish containing 4 ml 6xSSC and 60 pmol of the ^{32}p-labeled mutant oligonucleotide for 1 hour at room temperature, and rinsed in 100 ml 6xSSC. The filter was covered with Saranwrap and autoradiographed on preflashed films (Fuji) at -70 ° C for 1 hour. Subsequently, the filter was again washed in 6xSSC buffer at increasing temperatures (varying between 51 ° C and 75 ° C, according to the lenght of the probe and its amount of G and C residues), followed each time by an autoradiography, as described above. For instance, a wash at 64 ° C could clearly distinguish the Ser29 mutants from the wild-type colonies, while the Trp32 mutants were detected after two subsequent washes at 62 ° C and 63 ° C, respectively.

Restriction fragment analysis

Because the Ser29 mutation created an Ava2 restriction site and Arg32 destroyed the Nci1 restriction site, both corresponding endonucleases could be used for restriction fragment analysis to check once again the presence of the mutation. The colonies were picked up and grown to confluency in 5 ml + chloramphenicol. From these cultures, plasmid DNA was prepared, digested with the appropriate restriction endonucleases and electrophoresed on agarose gels, according to classical procedures (Sambrook et al., 1989).

Subcloning to a bacterial expression vector

Transfer of the mutated TNF gene to an expression vector was carried out exactly the opposite way as the construction of the mutagenesis vector. The phasmid pMc/RBSII,Sph1-TNFaSer29 or pMc/RBSII,Sph1-TNFαTrp32 was digested with EcoR1-HindIII and the small fragment was inserted into the EcoR1-HindIII opened pDS56/RBSII,Sph1-TNFα vector generating plasmids pDS56/RBSII,Sph1-TNFαSer29 and pDS56/RBSII,Sph1-TNFαTrp32and transformed into E. coli M15 cells containing already plasmid pREP4 (encoding the lac repressor; see Figures 2a and 2b/1-2b/3 for a complete nucleotide sequence of the plasmid given by the one letter standard abreviations for nucleotides) by standard methods (s.a.). Such cultures of transformed E. coli M15 were grown at 37 ° C in LB medium (10 g bacto tryptone, 5 g yeast extract, 5 g NaC1 per litre) containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2mM. After additional 2.5 to 5 h at 37 ° C the cells were harvested by centrifugation and the TNF muteins were purified according to Tavernier et al. [J. Mol. Biol. 211, 493-501, (1990)]. The transformed E. coli strains M15 (pREP4;pDS56/RBSII,Sph1-TNFαSer29) and M15(pREP4;pDS56/RBSII,Sph1-TNFαTrp32) have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH(DSM) in Braunschweig, BRD at November 19th, 1990 under accession numbers DSM 6240 and DSM 6241

respectively.

Example II

Characterization of Ser^{29}-TNFα and Trp^{32}-TNFα

a) Differential binding and biological activity on Hep2- and U937 cells

Cell culture

Hep-2 [ATCC No. CCL 23], U937 [ATCC No. CRL 1593] and RAJI [ATCC No. CCL 86] cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) inactivated fetal calf serum, L-glutamine (2mM), sodium pyruvate (1mM), 2-mercaptoethanol ($5x10^{-5}$ M), 1% of a 100x mixture of non-essential amino acids [Gibro Laboratories, Paisley, GB] and gentamycine (25 mg/ml). The non-adherent cells (U937 and RAJI) were harvested after reaching a density of $1x10^6$ cells/ml. For binding experiments, the adherent Hep-2 cells were grown to confluency, trypsinized, collected and seeded in large Petri dishes (150 cm^2) at a density of $2.5x10^6$ cells/ml. Subsequently, the dishes were placed in a CO_2-incubator overnight. Because Hep-2 cells are not strongly adherent, the cells could be harvested the same way as the non-adherent cells. Dulbecco's medium, supplemented with 10% inactivated newborn calf serum was used for L929 cell growth.

Determination of the specific activities on L929, Hep-2 and U937 cells.

The amount of protein was determined by the Biorad (Richmond, CA, USA) protein dye reagent according to the instructions of the manufacturer. The purity of the TNF muteins was determined by SDS-PAGE.

The cytotoxic activity on mouse L929 cells was determined using the standard L929 assay (Ruff and Gifford in "Lymphokines", ed. by E. Pick, Vol. 2, 235-275, Academic Press, 1981, Orlando, USA). The cytotoxicity assay on Hep-2 cells was performed the same way as the L929 assay with the only exception that cycloheximide (50 µg/ml) was added instead of actinomycin D.

Receptor binding assay

-Iodination of TNF-α and Trp^{32}-TNF

5 µg Iodogen (Pierce, USA) was dissolved in 10 µl chloroform and dried under a nitrogen stream in a small glass tube. To this, 10 µl $Na^{125}I$ (Amersham, 100 mCi/ml in 0.1 M borate buffer, pH 8) was added and kept for 15 min. on ice. This solution was quickly pipetted to an Eppendorf tube, containing 5 µg TNF-α - [Pennica et al., s.a.] or 3.2 µg of Trp^{32}-TNF in 10 µl phosphate buffer pH 7. Again the reaction was kept for 15 min on ice. To separate the iodinated TNF-α from the $Na^{125}I$, a PD-10 gelfiltration column (Pharmacia) was first equilibrated with 0.1 M phosphate buffer + 0.25% gelatin and prerun with 1 µg TNF-α or Trp^{32}-TNF, depending on the iodinated TNF species. Subsequently, the reaction mixture was loaded onto the column, and fractions of about 400 µl were collected from which 2 µl aliquots were counted in a γ-counter (LKB 1275 Minigamma, Pharmacia LKB, Uppsala, Sweden). A specific radioactivity of 10-75 and 80 µCi/mg was obtained for THF-α and Trp^{32}-TNF, respectively.

-Determination of the K_D-value of labeled TNF-α and Trp^{32}-TNF by Scatchard analysis

A dilution series in steps of factor 2 in the range of 12.8nM -> 0.006nM of the labeled TNF-α or Trp^{32}-TNF was made up in a microtiterplate. Each dilution was made in triplicate. Non-specific binding was measured by the same setup, wherein each point contained a 100 fold excess of unlabeled TNF (1.28 µM -> 0.6nM). To each well, approximately $2x10^6$ cells (U937, Hep-2 or RAJI) were added. The reaction was performed in 0.2 ml tissue culture medium, containing 0.1% NaN_3 for 2-3 hours at 4°C. After this, samples were transferred from the microtiterplates to small plastic tubes (Micronic systems), already containing 300 µl phthalate oil (dinonylphthalate 33%, dibutylphthalate 66% (v/v)). The tubes were centrifuged in a microfuge (Eppendorf) for 10 min. to spin down the cells, thereby separating them from the supernatant, using the phthalate oil as a separation medium. After inversion of the tubes, the cell pellet (now on top) could easily be isolated by melting off the top of the tubes with a hot scalpel. The amount of radioactivity,

bound on the cells, was measured by counting in a γ-counter. From these data, a Scatchard plot and, subsequently, the dissociation constant K_D was determined using the equilibrium binding type "HOT" in the EBDA/LIGAND programm [Mc.Pherson et al., J. Pharmacol. Methods 14, 213-228, (1985)].

-Determination of the K_D of mutant TNF [Ser^{29}-TNF-α and Trp^{32}-TNF-α] by competition analysis

The Scatchard data showed that a concentration of 0.4 nM radiolabeled TNF-α was high enough to show a clearly detectable signal and fell within the linear part of the saturation curves. This concentration, however, was also low enough to allow addition up to a 5000 fold excess of cold mutant TNF (2 μM), necessary to perform a competition experiment in which ^{125}I-wild type TNF is the primary ligand and cold mutant the competitor.

A ten well dilution series of unlabeled mutant TNF (2 mM -> 0.004 μM) in concentration steps of factors x2 was set up in a microtiterplate. The two remaining wells contained no unlabeled TNF (total binding) and a 5000 fold excess of the wild-type, unlabeled TNF (background), respectively. To all wells, 0.4 nM of radiolabeled TNF-α (10-75 μCi/μg) was added. After addition of $2x10^6$ cells, the total volume was 0.2 ml/well. Medium of incubation, reaction conditions and isolation of the cells were exactly the same as described above for the Scatchard analysis experiments. Each point was measured in triplicate and the dissociation experiments were done twice, the average of the two K_D's being indicated in Table 1. Using the "DRUG" method of the EBDA/LIGAND program (s.a.), competition curves were plotted and the K_D of the muteins was calculated. The following experimental data were used for such calculations:

1. Labeling of hTNF

first labeling (=batch 1):	$1.2x10^8$ dpm/5μg
	$=3.7x10^5$ dpm/pmol
	=±10μCi/μg
second labeling (=batch 2:)	$5.3x10^8$ dpm/3.2 μg
	$=1.9x10^6$ dpm/pmol
	=± 75 μCi/μg

2. Determination of the K_D of wild-type TNF

We measured the K_D of ^{125}I-TNF (batch 1) on Hep-2 and U937 cells by Scatchard analysis.
Hep-2: $K_D = 9.17x10^{-10}$
U937: $K_D = 2.5x10^{-10}$

3. Competition experiments

All displacement experiments were carried out, using ^{125}I-TNF (batch 1) as the primary ligand, except experiment B.3 (table B, 3.), where ^{125}I-TNF (batch 2) was used.

In each experiment, the binding at each concentration was measured in triplicate and only the averages are shown in the following tables (A-D).

From each experiment shown in these tables, the K_D value was calculated using the programm of Mc. Pherson et al. (1985). The average of the K_D determinations (2 experiments for Ser^{29}-TNFα on Hep-2 cells and on U937 cells, two experiments for Trp^{32}-TNFα on Hep-2 cells and three on U937 cells) are shown in table 1.

Table A

Competition with Ser^{29}-TNFα on U937 cells.

	Mean dpm	concentration of mutant [mol]
1.	2120	0
	1869	$1x10^{-9}$
	1779	$2x10^{-9}$
	1719	$4x10^{-9}$
	1708	$8x10^{-9}$
	1575	$1.6x10^{-8}$
	1415	$3.2x10^{-8}$
	1320	$6.4x10^{-8}$
	1200	$1.25x10^{-7}$
	983	$2.5x10^{-7}$
	949	$5x10^{-7}$
	632	$1x10^{-6}$
	533	$2x10^{-6}$
Background:	299	

2.	1014	0
	635	$4x10^{-9}$
	603	$8x10^{-9}$
	541	$1.5x10^{-8}$
	572	$3x10^{-8}$
	489	$6x10^{-8}$
	413	$1.2x10^{-7}$
	380	$2.5x10^{-7}$
	319	$5x10^{-7}$
	263	$1x10^{-6}$
	238	2.10^{-6}
Background:	205	

Table B

Competition with Trp^{32}-TNF-α on U937 cells

1.	2120	0
	1917	$1x10^{-9}$
	1698	$2x10^{-9}$
	1655	$4x10^{-9}$
	1585	$8x10^{-9}$
	1488	$1.5x10^{-8}$
	1377	$3x10^{-8}$
	1333	$6x10^{-8}$
	1166	$1.25x10^{-7}$
	1026	$2.5x10^{-7}$
	953	$5x10^{-7}$
	777	$1x10^{-6}$
	628	$2x10^{-6}$
Background:	299	

2.	1047	0
	653	$4x10^{9}$
	629	$8x10^{-9}$
	636	$1.5x10^{-8}$
	585	$3x10^{-8}$
	546	$6x10^{-8}$

	508	$1.2x10^{-7}$
	479	$2.5x10^{-7}$
	422	$5x10^{-7}$
	357	1.10^{-6}
	294	$2x10^{-6}$
Background:	214	
3.	8340	0
(carried out	4759	$4x10^{-9}$
with ^{125}I-	4041	$8x10^{-9}$
TNF, batch 2)	3620	$1.5x10^{-8}$
	3275	$3x10^{-8}$
	3034	$6x10^{-8}$
	2387	$1.25x10^{-7}$
	1981	$2.5x10^{-7}$
	1472	$5x10^{-7}$
	1192	$1x10^{-6}$
	814	$2x10^{-6}$
Background:	307	

Table C

Competition with Ser^{29}-TNF-α on Hep-2 cells

1.	938	0
	799	$1x10^{-9}$
	677	$2x10^{-9}$
	564	$4x10^{-9}$
	510	$8x10^{-9}$
	451	$1.6x10^{-8}$
	442	$3.2x10^{-8}$
	446	$6.4x10^{-8}$
	379	$1.25x10^{-7}$
	374	$2.5x10^{-7}$
	437	$5x10^{-7}$
	359	$1x10^{-6}$
	383	$2x10^{-6}$
Background:	353	

2.	457	0
	273	$4x10^{-9}$
	240	$8x10^{-9}$
	253	$1.5x10^{-8}$
	235	$3x10^{-8}$
	207	$6x10^{-8}$
	239	$1.2x10^{-7}$
	215	$2.5x10^{-7}$
	211	$5x10^{-7}$
	193	$1x10^{-6}$
	238	$2x10^{-6}$
Background:	215	

Table D

Competition with Trp^{32}-TNF-α on Hep-2 cells

1.	938	0
	742	$1x10^{-9}$
	608	$2x10^{-9}$
	537	$4x10^{-9}$
	547	$8x10^{-9}$
	397	$1.6x10^{-8}$
	394	$3.2x10^{-8}$
	405	$6.4x10^{-8}$
	395	$1.25x10^{-7}$
	388	$2.5x10^{-7}$
	379	$5x10^{-7}$
	353	$1x10^{-6}$
	386	$2x10^{-6}$
Background:	353	
2.	445	0
	298	$4x10^{-9}$
	222	$8x10^{-9}$
	256	$1.5x10^{-8}$

	202	$3x10^{-8}$
	227	$6x10^{-8}$
	210	$1.2x10^{-7}$
	221	$2.5x10^{-7}$
	197	$5x10^{-7}$
	231	$1x10^{-6}$
	202	$2x10^{-6}$
Background:	203	

Table 1

	Hep-2		U937	L929
	affinity (K_D)	specific activity (U/mg)	affinity (K_D)	specific activity (U/mg)
TNF-α	$9.17x10^{-10}$(*) (100%)	$2.9x10^{7}$ (100%)	$2.5x10^{-10}$(*) (100%)	$2x10^{7}$ (100%)
Ser^{29}-TNF-α	$1.06x10^{-9}$ (86.5%)	$9.3x10^{6}$ (32%)	$5.07x10^{-8}$ (0.49%)	10^{5} (0.5%)
Trp^{29}-TNF-α	$1.06x10^{-9}$ (86.5%)	$4.5x10^{7}$ (155%)	$3.53x10^{-8}$ (0.71%)	$6.4x10^{4}$ (0.32%)

K_D values indicated by an asterisk (*) were obtained by Scatchard analysis. All other K_D values were determined by competition analysis. Relative values (in percentage to TNF-α) are indicated between brackets.

It can be seen that the binding constant (K_D) of Ser^{29}-TNF-α and Trp^{32}-TNF-α determined with Hep-2 cells (which only carry the p55-TNF-R) are almost the same as the one of TNF-α. Also the biological activity (specific activity) on these cells is largely retained (note that the accuracy of this assay is only a factor 3). Strikingly, the binding affinity (measured in the competition assay) of Ser^{29}-TNF-α and Trp^{32}-TNF-α to the U937 cells, which predominantly - but not exclusively - carry the high affinity receptor p75-TNF-R, has been largely lost (increase in K_D-value by a factor of more than 100). It may also be noted that the biological activity of Ser^{29}-TNF-α and Trp^{32}-TNF-α, determined in the standard assay based on L929-cells, has been largely lost (decrease by a factor more than 100).

b) Differential binding to the human p75-TNF-R and the human p55-TNF-R.

Competition of human ^{125}I-TNF-α binding by Trp^{32}- and Ser^{29}-TNF-α and human TNF-α to TNF-receptors purified from HL60 cells was determined as follows. 2 µl aliquots of the native p55-TNF-R and the p75-TNF-R purified as described in European Patent Application No. 90116707.2 dissolved at a concentration of about 0.3 mg/ml in 20 mM Hepes, 50 mM Tris, 50 mM NaCl, 1 mM EDTA, 0.1% octylglucoside, 0.1 mg/ml BSA, pH 8.0, were spotted onto prewetted nitrocellulose filters in triplicate. The filters were blocked with blocking buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1% defatted milk powder) for 1.5 hours at room temperature. After washing with PBS the filters were incubated with 10 ng/ml ^{125}I TNFα and varying concentrations of Trp^{32}- or Ser^{29}-TNFα, or TNFα overnight at 4 °C. The filters were washed with blocking buffer (2x for 5 min.) and with H_2O (1x for 5 min.), air dried, and counted in a γ-counter. Results

are given in Figures 1a and b, whereby Figure 1 shows binding of TNFα (open rectangle), Ser^{29}-TNFα - (filled circles) and Trp^{32}-TNFα (filled rectangle) to human p75TNF-R in case of Figure 1a to human p75-TNF-R and in case of Figure 1b to human p55-TNF-R.

Example III

Purification of Trp^{32}-TNFα

Transformed cells obtained according to Example I were processed in the following manner:
a) Opening by French press, addition of polyethylene-imine until a final concentration of 0.4%, pH 7.6; removal of precipitate.
b) Ammonium sulphate precipitation at pH 7.2; fraction 30-70%
c) Dialysis against 25% ammonium sulphate in 10 mM Tris, pH 6.8
d) Phenyl-Sepharose column CL-4B (35 x 250 mm)
Load in 25% ammonium sulphate - 10 mM Tris, pH 6.8
Elution: gradient 25% ammonium sulphate-Tris buffer to 20 mM ethanolamine, pH 9 (2 times 150 ml).
e) Column Mono Q (HR 16/10).
Load: in 20 mM ethanolamine, pH 9. Elution: gradient (2 times 300 ml) in the same buffer, from 0 to 1 M sodium chloride (Pharmacia, FPLC). Active fractions dialysed versus 0.01 M phosphate buffer pH 7
f) Column of Heparin Sepharose CL-6B (30 x 80 mm)
Load in 0.01 M phosphate buffer pH 7. Elute with a gradient in the same buffer from 0 to 1 M sodium chloride
g) Active fractions were concentrated on Amicon (micro-ultrafiltration system 8 MC; membrane O 25 mm; diaflo 10 YM10 - 25 mm) and separately loaded on a gelfiltration column (Ultrapac TSK G-2000 SWG; 21.5 x 600 mm), equilibrated in 0.01 M phosphate pH 7 and 0.9% sodium chloride
LPS (determined by test kit of Kabivitrum):
Most active fraction contained 5 mg/ml Trp^{32}-TNFα; endotoxin content: 26 E.U./mg
The last active fraction contained 1.8 mg/ml TNF and 47 E.U./mg protein.

1. Anti-tumour effect of hTNFα and $hIFN_\gamma$ on subcutaneous HT-29 tumours in nude mice.

$5x10^6$ HT-29 human colon adenocarcinoma cells [ATCC HTB38] were subcutaneously injected in nude mice. Groups consisted of 5 mice. The treatment comprises daily perilesional injections during 6 days per week, followed by 1 day without treatment. Results are given in Fig. 4 whereby "PBS" refers to phosphate buffered saline as known in the art. The single arrow indicates the start of the treatment with 5 µg hTNFα or 5000 IU human Interferon γ ($hIFN_\gamma$) or both. The double arrow indicates the time that these doses were doubled and the crossed arrow indicates the end of the treatment.

2. Comparison of the anti-tumour potential of hTNFα and Trp^{32}-

$5x10^6$ HT-29 human colon adenocarcinoma cells (s.a.) were subcutaneously injected in nude mice. Groups consisted of 5 mice. The treatment started on day 6 following inoculation and comprises daily perilesional injections during 6 days per week. Tumour volume was estimated every 3 or 4 days by measuring the larger (a) and the smaller (b) diameter and calculating the $a \times b^2 \times 0.4$ according to Attia and Weiss as known in the art. Results are given in Fig. 5 whereby the arrow indicates the start of the treatment and open triangles with tip down refers to 10^4 IU of $hIFN_\gamma$ and 10 µg hTNFα, filled triangles with tip down refer to 10^4 IU of hIFN-γ and 10 µg Trp^{32}-TNF, filled rectangles refer to 10 µg Trp^{32}-TNFα, open reactangles refer to 10 µg hTNFα, open triangles refer to phosphate bufferred saline and filled circles refer to 10^4 IU of $hIFN_\gamma$. In vitro, there is no difference in cytotoxicity for Hep or HT-29 cells between hTNFα and Trp^{32}-TNFα.

Example V

Preparation of Ser^{29}-Trp^{32}-TNFα

Ser^{29}-Trp^{32}-TNFα was prepared as described in Example I with the following exceptions:
1. The oligonucleotide used, contains the following sequence (mutated bases underlined):
5'GGGCATTGGCCCAGCGGTTGGACCACTGGAGC3'

2. An Nci 1 site was destroyed while an Ava 2-site was created, allowing for check of the presence of the mutation by restriction fragment analysis. No hybridization analysis was performed. 6 clones resulting from the WK6 transformation were grown up and DNA was prepared and analysed as described in Example I. 3 from 6 clones beared the mutation.

This DNA sequence was subcloned into the pDS56 expression vector, generating the plasmid pDS56/RBSII,Sph1-TNFαSer29Trp32, and transformed to the E. coli M15 strain. Expression and purification was performed as described in Example I.

Example VI

Preparation of Gly^{29}-TNFα,Tyr^{29}-TNFα and Tyr^{32}-TNFα

Gly^{29}-TNFα,Tyr^{29}-TNFα and Tyr^{32}-TNFα were prepared as described in Example I with the following exception. Oligonucleotides were used, containing a fully degenerated codon at position 29 or 32, resulting in a random insertion of all twenty amino acids at one of the two positions. The sequence of these oligonucleotides are as follows:

Position 29:

5' CCACGCCATTCGCGAGGAGGGCATTGGCCCGGCGGTTXXXCCACTGGAGC 3'

Position 32:

5' CCACGCCATTCGCGAGGAGGGCATTGGCXXXGCGGTTCAGCC 3'

where X = A, C, G or T and mutated bases are underlined.

Together with the mutation, also a unique Nru-1 site is introduced. Thus, instead of directly transforming the phasmid-pool, isolated from the WK6 mutS strain, this DNA was first digested with Nru-1, the linear band eluted from the agarose gel, ligated and transformed to the SURE-strain (Stratagene, La Jolla, CA, USA). In this way, one can select only for phasmids, containing the mutations. 168 colonies obtained were inoculated in microtiterplates, grown to confluency and their lysates tested for biological activity towards Hep-2 cells in a manner as described in Example IIa and for differential binding as described in Example IIb or Example VIII. On the basis of the biological activity on the one side and differential binding as determined according to Example IIb or Example VIII colonies were selected and further characterized by DNA sequence analysis of corresponding inserts as known in the art. DNA-sequences coding for Gly^{29}-TNFα, Tyr^{29}-TNFα and Tyr^{32}-TNFα were isolated from corresponding colonies and cloned in bacterial expression vectors as described in Example I. Muteins expressed were purified to more than 95% homogeneity by means of a MONO-Q ion exchange chromatography step.

Example VII

Preparation of Glu^{31}-TNFα and Asn^{31}-Thr^{32}-TNFα

Mutagenesis of the TNFα gene using PCR

Three PCR reactions were performed with plasmid pDS56/RBSII,Sph1-TNFα [Figure 3] as the template DNA using a Perkin-Elmer Cetus GeneAmp™ DNA Amplification Reagent Kit with AmpliTaq™ Recombinant Taq DNA Polymerase (Perkin Elmer Cetus, Vaterstetten, BRD) [see Figure 8]. In reaction I primers 17/F (5'-GGCGTATCACGAGGCCCTTTCG-3'; primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,SphI-TNFα) and 21/M5 (5-ATTGGCCCGCTCGTTCAGCCACTGGAGCTGCCCCTC-3'; primer 21/M5 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,Sph1-TNFα, mutated bases are underlined) were used , reaction II contained primers 17/F and 21/M6 (5'-ATTGGCAGTGTTGTTCAGCCACTGGAGCTGCCCCTC-3'; primer 21/M6 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,Sph1-TNFα, mutated bases are underlined), and reaction III contained primers 21/MR (5'-GCCCTCCTGGCCAATGGCGTGG-3'; primer

21/MR comprises nucleotides 220-241 of plasmid pDS56/RBSII,Sph1-TNFα) and 17/O (5'-CATTACTGGATCTATCAACAGG-3'; primer 17/O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,Sph1-TNFα). Therfore 10 µl template DNA (10 ng), 5 µl each of the two primers (100 pmole each), 16 µl dNTP's mix (1.25 mM of dATP, dGTP, dCTP, and dTTP), 10 µl 10x reaction buffer (100 mM Tris-HCl pH8.3, 500 mM KCL, 15 mM $MgCl_2$ and 0.1 % gelatin), 1 µl (5 units) AmpliTaq™ DNA polymerase and 53 µl H_2O were mixed in an Eppendorf tube and overlaid with 80 µl mineral oil (Perkin-Elmer Cetus). The tubes were transferred to a DNA thermal cycler (TRIO-Thermoblock, Biometra) and kept for 1 min at 94°C, before 35 cycles of melting the DNA (1 min at 94°C), annealing the primers (1 min at 50°C),and extending the primers (3 min at 72°C) were performed. After additional 2 min at 72°C, the reactions were cooled to room temperature and extracted with chloroform. The DNA present in the aqueous phase was precipitated with ethanol and subjected to electrophoresis in a 6 % polyacrylamide gel [Sambrook et al., 1989]. After staining of the DNA with ethidium bromide, fragments I, II and III [see Figure 8; these fragments originate from reactions I, II and III, respectively] were isolated from the gel and purified [Sambrook et al.. 1989].

Preparation of DNA fragments encoding Glu^{31}-TNFα and Asn^{31}-Thr^{32}-TNFα

Fragments I, II and III were enzymatically phosphorylated, before in two parallel reactions fragments I and III and fragments II and III were ligated with each other [Sambrook et al., 1989]. After heat-inactivation of the ligase and digestion with restriction enzymes EcoRI and HindIII, the DNA was subjected to electrophoresis in a 6 % polyacrylamide gel. After staining of the DNA with ethidium bromide, the EcoRI-HindIII fragments A and B [see Figure 4] were isolated from the gel and purified [s.a].

Preparation of plasmids encoding Glu^{31}-TNFα and Asn^{31}-Thr^{32}-TNFα

In separate experiments, the EcoRI-HindIII fragments A and B were inserted according to standard methods [Sambrook et al., 1989] into the EcoRI-HindIII opened plasmid pDS56/RBSII,Sph1-TNFαSer29 generating plasmids pDS56/RBSII,Sph1-TNFαGlu31 and pDS56/RBSII,Sph1-TNFαAsn31Thr32, respectively. Plasmid DNA was prepared [Birnboim et al., 1979] and the identity of the coding region for the TNFα muteins was confirmed by sequencing the double-stranded DNA [Sambrook et al., 1989].

Production of Glu^{31}-TNFα and Asn^{31}-Thr^{32}-TNFα

Plasmids pDS56/RBSII,Sph1-TNFαGlu31 and pDS56/RBSII,Sph1-TNFαAsn31Thr32 were transformed into E. coli M15 cells containing already plasmid pREP4 by standard methods [s.a.]. Transformed cells were grown at 37°C in LB medium [s.a.] containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2 mM. After additional 2.5 to 5 h at 37°C the cells were harvested by centrifugation.

Example VIII

Differential binding to recombinant human p75-TNF-R and recombinant human p55-TNF-R

1. 10 ml suspensions of transformed and induced E. coli cells expressing recombinant human TNFα, Ser^{29}-TNFα, Trp^{32}-TNFα, Glu^{31}-TNFα, and Asn^{31}-Thr^{32}-TNFα [E. coli cells expressing recombinant dihydrofolate reductase (DHFR) were included as a control] were centrifuged at 4'000 rpm for 10 min and resuspended in 0.9 ml of lysis buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 200 units/ml aprotinine and 0.1 mg/ml lysozyme). After 20 min incubation at room temperature 50 µl of 1 M $MgCl_2$, 20 µl of 5 mg/ml DNaseI, 50 µl of 5 M NaCl and 50 µl of 10% NP-40 were added and the mixture was further incubated at room temperature for 15 min. 0.5 ml of the lysate clarified by centrifugation at 13'000 rpm for 5 min was subjected to ammonium sulfate precipitation (30% - 70% cut). The 70% ammonium sulfat pellet was dissolved in 0.2 ml PBS and analyzed by SDS-PAGE to confirm the presence of the recombinant proteins.

For the differential binding assay microtiter plates were coated with recombinant human p75-TNF-R-human IgGγ3 and p55-TNF-R-human IgGγ3 fusion proteins (European Patent Applications Publ. Nos. 417 563, 422 339) dissolved in PBS at 0.3 µg/ml and 0.1 µg/ml, respectively, (100 µl/well, overnight at 4°C). After blocking with blocking buffer (50 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1% defatted milk powder) the microtiter plate was washed with PBS and incubated with 5 ng/ml human

^{125}I-TNFα (labelled by the Iodogen method to a specific activity of about 30 μCi/μg as described above) in the presence of different dilutions of the E. coli lysate partially purified by ammonium sulfate precipitation. The volume was 100 μl/well and each dilution was assayed in duplicate. After three hours at room temperature the wells were thoroughly washed with PBS and counted in a γ-counter. Results are shown in Fig.6 whereby closed circles refer to binding to p55-TNF-R-human IgGγ3-and open circles refer to binding to p75-TNF-R-human IgGγ3.

2. Determination of binding of Ser^{29}-Trp^{32}-TNFα, Gly^{29}-TNFα, Tyr^{29}-TNFα and Tyr^{32}-TNFα was performed as described under 1. with the only exception that MONO-Q ion exchange chromatography purified muteins were used. Results are shown in Fig. 7 whereby open and closed circles have the same meaning as in Fig. 6 and μg/ml gives the amount of purified mutein/ml.

Claims

1. A human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor.

2. A mutein as claimed in claim 1 or a pharmaceutically acceptable salt thereof wherein the amino acid sequence of human Tumor Necrosis Factor is

```
 1                                                  10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

                    20                                  30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

                                                    40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

                    50                                  60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

                                                    70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

                    80                                  90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

                                                   100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

                   110                                 120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

                                                   130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU

                   140                                 150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL

                                                   157
TYR PHE GLY ILE ILE ALA LEU.
```

3. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed by substitution of one or more, preferably one or two amino acids by other amino

acids, preferably naturally occuring amino acids.

4. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 29 as claimed in claim 3.

5. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occuring amino acid is serine.

6. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is glycine.

7. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is tyrosine.

8. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 32 as claimed in claim 3.

9. A mutein as claimed in claim 8 or a pharmaceutically acceptable salt thereof wherein said naturally occuring amino acid is tryptophan.

10. A mutein as claimed in claim 8 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is tyrosine.

11. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 31 as claimed in claim 3.

12. A mutein as claimed in claim 11 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is glutamic acid.

13. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at positions 29 and 32 as claimed in claim 3.

14. A mutein as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid at position 29 is serine, glycine or tyrosine, preferably serine and at position 32 tyrosine or tryptophase, preferably tryptophan.

15. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at positions 31 and 32.

16. A mutein as claimed in claim 15 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid at position 31 is glutamic acid or asparagine, preferably asparagine and at position 32 tyrosine, tryptophan or threonine, preferably threonine.

17. A deletion, substitution and/or addition analog of a mutein as claimed in any one of claims 4-16 or a pharmaceutically acceptable salt thereof whereby position 29 and/or 32 or position 31 or position 31 and 32 of the mutein is/are not changed and which analog still shows a significant difference between its binding affinity to the human p75-Tumor Necrosis-Factor-Receptor and the human p55-Tumor Necrosis-Factor-Receptor.

18. A DNA-sequence comprising a DNA-sequence coding for a mutein as claimed in any one of claims 1-17.

19. A vector, especially for expression in a procaryotic or lower eukaryotic host cell, such vector comprising a DNA-sequence as claimed in claim 18.

20. A host cell, especially a procaryotic or lower eukaryotic host cell transformed by a vector as claimed in claim 19.

21. A host cell as claimed in claim 20 which is E. coli.

22. A compound as claimed in any one of claims 1-17 for the treatment of illnesses.

23. A process for the preparation of a compound as claimed in any one of claims 1-17 which process comprises cultivating a host cell as claimed in claim 20 or claim 21 in a suitable medium and isolating the mutein from the culture supernatant or the host cell itself, and if desired converting said mutein into a pharmaceutically acceptable salt.

24. A pharmaceutical composition which contains one or more compounds as claimed in any one of claims 1-17, if desired, in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

25. The use of a compound as claimed in any one of claims 1-17 for the treatment of illnesses.

Claims for the following Contracting States : GR,ES

1. A process for the preparation of a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof which mutein is characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor which process comprises cultivating a host cell transformed with an expression vector comprising a DNA sequence coding for such a mutein in a suitable medium and isolating this mutein from the culture supernatant or the host cell itself, and if desired converting this mutein into a pharmaceutically acceptable salt.

2. A process as claimed in claim 1 whereby the amino acid sequence of human Tumor Necrosis Factor is

```
 1                                                  10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

                   20                                                 30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

                                                   40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

                   50                                                 60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

                                                   70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

                   80                                                 90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

                                                  100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

                  110                                                120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

                                                  130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU
```

```
                    140                                                 150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL

                        157
TYR PHE GLY ILE ILE ALA LEU.
```

3. A process as claimed in claim 2 whereby said amino acid sequence is changed by substitution of one or more, preferably one or two amino acids by other amino acids, preferably naturally occuring amino acids.

4. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 29 as claimed in claim 3.

5. A process as claimed in claim 4 whereby said naturally occuring amino acid is serine.

6. A process as claimed in claim 4 whereby said naturally occurring amino acid is glycine.

7. A process as claimed in claim 4 whereby said naturally occurring amino acid is tyrosine.

8. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 32 as claimed in claim 3.

9. A process as claimed in claim 8 whereby said naturally occuring amino acid is tryptophan.

10. A process as claimed in claim 8 whereby said naturally occurring amino acid is tyrosine.

11. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 31 as claimed in claim 3.

12. A process as claimed in claim 11 whereby said naturally occurring amino acid is glutamic acid.

13. A process as claimed in claim 2 whereby said amino acid sequence is changed at positions 29 and 32 as claimed in claim 3.

14. A process as claimed in claim 13 whereby said naturally occurring amino acid at position 29 is serine, glycine or tyrosine, preferably serine and at position 32 tyrosine or tryptophane, preferably tryptophan.

15. A process as claimed in claim 2 whereby said amino acid sequence is changed at positions 31 and 32.

16. A process as claimed in claim 15 whereby said naturally occurring amino acid at position 31 is glutamic acid or asparagin, preferably asparagine and at position 32 tyrosine, tryptophan or threonine, preferably threonine.

17. A process for the preparation of a deletion, substitution and/or addition analog of a mutein obtained by a process as claimed in any one of claims 4-16 or a pharmaceutically acceptable salt thereof whereby position 29 and/or 32 or position 31 or position 31 and 32 of said mutein is/are not changed and which analog still shows a significant difference between its binding affinity to the human p75-Tumor Necrosis-Factor-Receptor and the human p55-Tumor Necrosis-Factor-Receptor which process comprises cultivating a host cell transformed with an expression vector comprising a DNA sequence coding for such an analog in a suitable medium and isolating the analog from the culture supernatant or the host cell itself, and if desired converting said analog into a pharmaceutically acceptable salt.

18. A process as claimed in any one of claims 1-17 whereby the host cell is a procaryotic or lower eukaryotic host cell.

19. A process as claimed in claim 18 whereby the prokaryotic host cell is E. coli.

20. A process as claimed in any one of claims 1-19 whereby the expression vector is a vector of the pDS family.

21. A process for the preparation of a pharmaceutical composition which process is characterized in that a compound obtained by a process as claimed in any one of claims 1-20 and if desired, additional pharmaceutically active substances are mixed with a non-toxic, inert, therapeutically compatible carrier material and the mixture is brought into a galenical application form.

22. A pharmaceutical composition which contains one or more compounds obtained according to a process as claimed in any one of claims 1-20, if desired, in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

23. The use of a compound prepared according to a process as claimed in any one of claims 1-20 for the preparation of a pharmaceutical composition according to claim 22.

24. A compound whenever prepared according to a process as claimed in any one of claims 1-20.

25. The invention as hereinbefore described.

26. A DNA-sequence comprising a DNA-sequence coding for a compound prepared according to a process as claimed in any one of claims 1-20.

27. A vector, especially for expression in a procaryotic or lower eukaryotic host cell, such vector comprising a DNA-sequence as claimed in claim 26.

28. A host cell, especially a procaryotic or lower eukaryotic host cell transformed by a vector as claimed in claim 27.

29. A host cell as claimed in claim 28 which is E. coli.

Fig. 1a

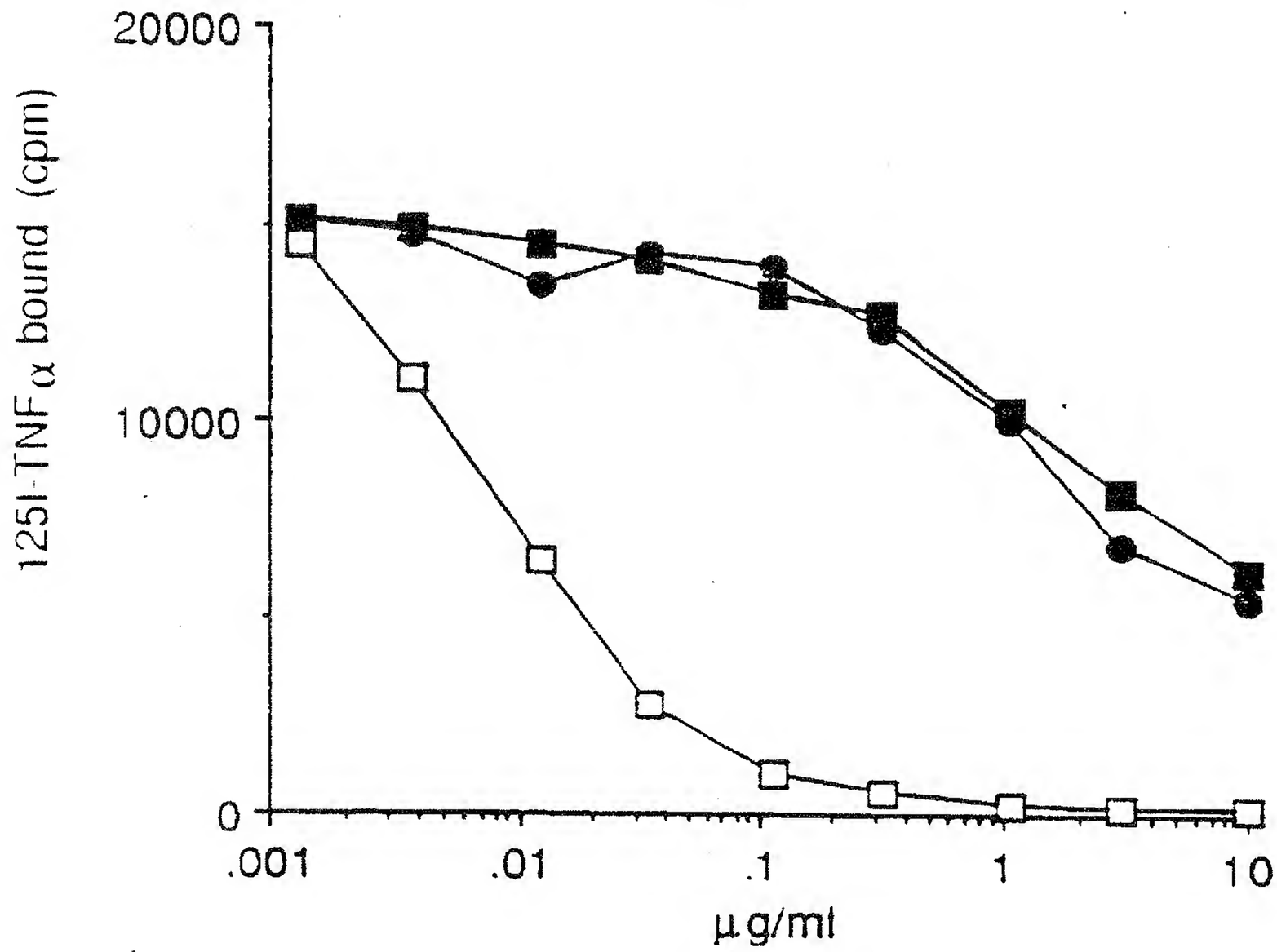


Fig. 1b

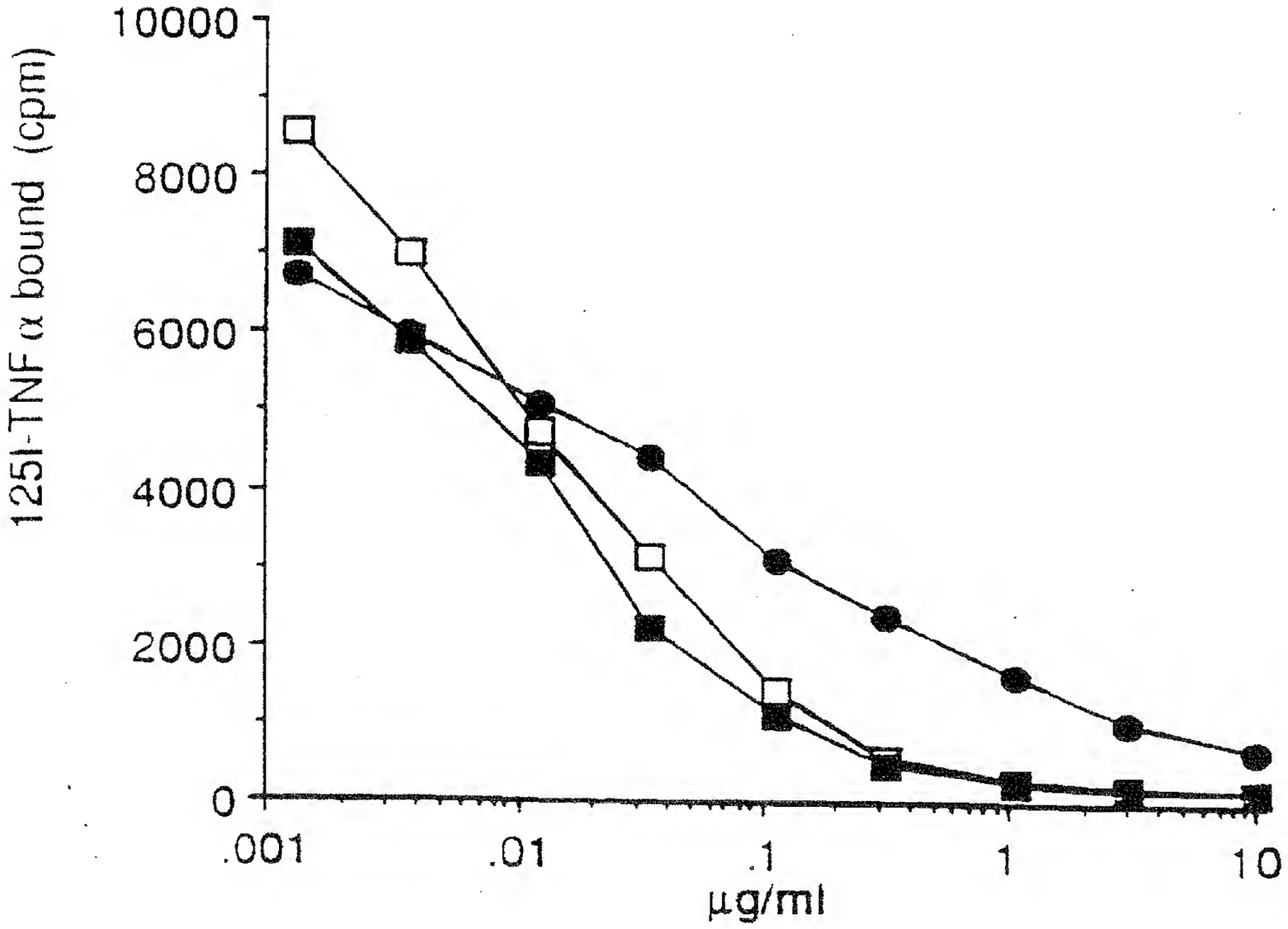


Fig. 2a

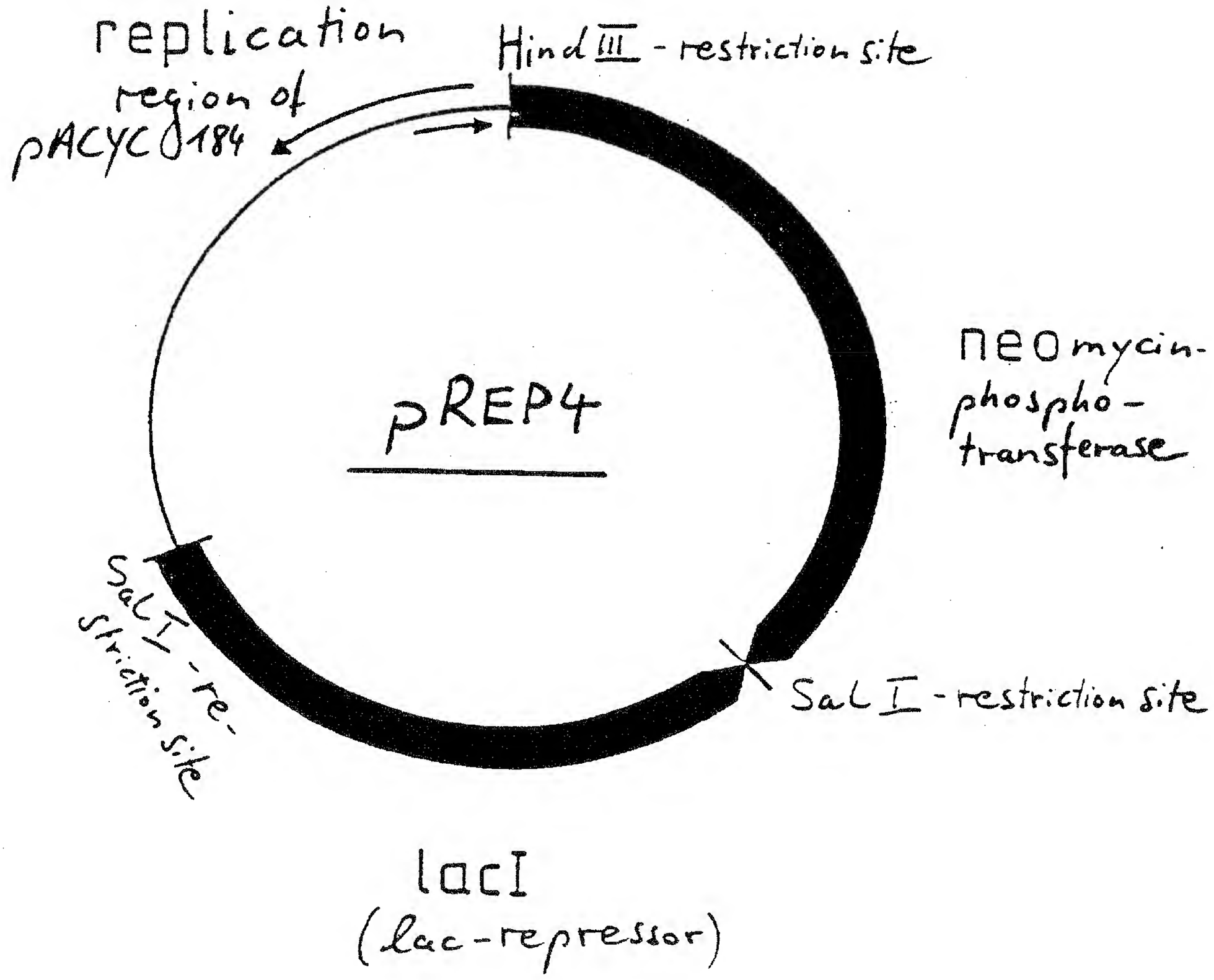


Fig. 2b/1

```
                HindIII
   1  AAGCTTCACG CTGCCGCAAG CACTCAGGGC GCAAGGGCTG CTAAAGGAAG
  51  CGGAACACGT AGAAAGCCAG TCCGCAGAAA CGGTGCTGAC CCCGGATGAA
 101  TGTCAGCTAC TGGGCTATCT GGACAAGGGA AAACGCAAGC GCAAAGAGAA
 151  AGCAGGTAGC TTGCAGTGGG CTTACATGGC GATAGCTAGA CTGGGCGGTT
 201  TTATGGACAG CAAGCGAACC GGAATTGCCA GCTGGGGCGC CCTCTGGTAA
 251  GGTTGGGAAG CCCTGCAAAG TAAACTGGAT GGCTTTCTTG CCGCCAAGGA
 301  TCTGATGGCG CAGGGGATCA AGATCTGATC AAGAGACAGG ATGACGGTCG
 351  TTTCGCATGC TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG
 401  GGTGGAGAGG CTATTCGGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT
 451  CTGATGCCGC CGTGTTCCGG CTGTCAGCGC AGGGGCGCCC GGTTCTTTTT
 501  GTCAAGACCG ACCTGTCCGG TGCCCTGAAT GAACTGCAGG ACGAGGCAGC
 551  GCGGCTATCG TGGCTGGCCA CGACGGGCGT TCCTTGCGCA GCTGTGCTCG
 601  ACGTTGTCAC TGAAGCGGGA AGGGACTGGC TGCTATTGGG CGAAGTGCCG
 651  GGGCAGGATC TCCTGTCATC TCACCTTGCT CCTGCCGAGA AAGTATCCAT
 701  CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC
 751  CATTCGACCA CCAAGCGAAA CATCGCATCG AGCGAGCACG TACTCGGATG
 801  GAAGCCGGTC TTGTCGATCA GGATGATCTG GACGAAGAGC ATCAGGGGCT
 851  CGCGCCAGCC GAACTGTTCG CCAGGCTCAA GGCGCGCATG CCCGACGGCG
 901  AGGATCTCGT CGTGACCCAT GGCGATGCCT GCTTGCCGAA TATCATGGTG
 951  GAAAATGGCC GCTTTTCTGG ATTCATCGAC TGTGGCCGGC TGGGTGTGGC
1001  GGACCGCTAT CAGGACATAG CGTTGGCTAC CCGTGATATT GCTGAAGAGC
1051  TTGGCGGCGA ATGGGCTGAC CGCTTCCTCG TGCTTTACGG TATCGCCGCT
1101  CCCGATTCGC AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG
1151  AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC CCAACCTGCC
1201  ATCACGAGAT TTCGATTCCA CCGCCGCCTT CTATGAAAGG TTGGGCTTCG
1251  GAATCGTTTT CCGGGACGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC
1301  ATGCTGGAGT TCTTCGCCCA CCCCGGGCTC GATCCCCTCG CGAGTTGGTT
```

Fig. 2b/2

```
1351  CAGCTGCTGC CTGAGGCTGG ACGACCTCGC GGAGTTCTAC CGGCAGTGCA
1401  AATCCGTCGG CATCCAGGAA ACCAGCAGCG GCTATCCGCG CATCCATGCC
                                        SalI
1451  CCCGAACTGC AGGAGTGGGG AGGCACGATG GCCGCTTTGG TCGACAATTC
1501  GCGCTAACTT ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
1551  GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA
1601  GGCGGTTTGC GTATTGGGCG CCAGGGTGGT TTTTCTTTTC ACCAGTGAGA
1651  CGGGCAACAG CTGATTGCCC TTCACCGCCT GGCCCTGAGA GAGTTGCAGC
1701  AAGCGGTCCA CGCTGGTTTG CCCCAGCAGG CGAAAATCCT GTTTGATGGT
1751  GGTTAACGGC GGGATATAAC ATGAGCTGTC TTCGGTATCG TCGTATCCCA
1801  CTACCGAGAT ATCCGCACCA ACGCGCAGCC CGGACTCGGT AATGGCGCGC
1851  ATTGCGCCCA GCGCCATCTG ATCGTTGGCA ACCAGCATCG CAGTGGGAAC
1901  GATGCCCTCA TTCAGCATTT GCATGGTTTG TTGAAAACCG GACATGGCAC
1951  TCCAGTCGCC TTCCCGTTCC GCTATCGGCT GAATTTGATT GCGAGTGAGA
2001  TATTTATGCC AGCCAGCCAG ACGCAGACGC GCCGAGACAG AACTTAATGG
2051  GCCCGCTAAC AGCGCGATTT GCTGGTGACC CAATGCGACC AGATGCTCCA
2101  CGCCCAGTCG CGTACCGTCT TCATGGGAGA AAATAATACT GTTGATGGGT
2151  GTCTGGTCAG AGACATCAAG AAATAACGCC GGAACATTAG TGCAGGCAGC
2201  TTCCACAGCA ATGGCATCCT GGTCATCCAG CGGATAGTTA ATGATCAGCC
2251  CACTGACGCG TTGCGCGAGA AGATTGTGCA CCGCCGCTTT ACAGGCTTCG
2301  ACGCCGCTTC GTTCTACCAT CGACACCACC ACGCTGGCAC CCAGTTGATC
2351  GGCGCGAGAT TTAATCGCCG CGACAATTTG CGACGGCGCG TGCAGGGCCA
2401  GACTGGAGGT GGCAACGCCA ATCAGCAACG ACTGTTTGCC CGCCAGTTGT
2451  TGTGCCACGC GGTTGGGAAT GTAATTCAGC TCCGCCATCG CCGCTTCCAC
2501  TTTTTCCCGC GTTTTCGCAG AAACGTGGCT GGCCTGGTTC ACCACGCGGG
2551  AAACGGTCTG ATAAGAGACA CCGGCATACT CTGCGACATC GTATAACGTT
2601  ACTGGTTTCA CATTCACCAC CCTGAATTGA CTCTCTTCCG GGCGCTATCA
2651  TGCCATACCG CGAAAGGTTT TGCGCCATTC GATGGTGTCA ACGTAAATGC
                                  SalI
2701  ATGCCGCTTC GCCTTCGCGC GCGAATTGTC GACCCTGTCC CTCCTGTTCA
```

Fig. 2b/3

2751 GCTACTGACG GGGTGGTGCG TAACGGCAAA AGCACCGCCG GACATCAGCG

2801 CTAGCGGAGT GTATACTGGC TTACTATGTT GGCACTGATG AGGGTGTCAG

2851 TGAAGTGCTT CATGTGGCAG GAGAAAAAAG GCTGCACCGG TGCGTCAGCA

2901 GAATATGTGA TACAGGATAT ATTCCGCTTC CTCGCTCACT GACTCGCTAC

2951 GCTCGGTCGT TCGACTGCGG CGAGCGGAAA TGGCTTACGA ACGGGGCGGA

3001 GATTTCCTGG AAGATGCCAG GAAGATACTT AACAGGGAAG TGAGAGGGCC

3051 GCGGCAAAGC CGTTTTTCCA TAGGCTCCGC CCCCCTGACA AGCATCACGA

3101 AATCTGACGC TCAAATCAGT GGTGGCGAAA CCCGACAGGA CTATAAAGAT

3151 ACCAGGCGTT TCCCCCTGGCG GCTCCCTCGT GCGCTCTCCT GTTCCTGCCT

3201 TTCGGTTTAC CGGTGTCATT CCGCTGTTAT GGCCGCGTTT GTCTCATTCC

3251 ACGCCTGACA CTCAGTTCCG GGTAGGCAGT TCGCTCCAAG CTGGACTGTA

3301 TGCACGAACC CCCCGTTCAG TCCGACCGCT GCGCCTTATC CGGTAACTAT

3351 CGTCTTGAGT CCAACCCGGA AGACATGCA AAAGCACCAC TGGCAGCAGC

3401 CACTGGTAAT TGATTTAGAG GAGTTAGTCT TGAAGTCATG CGCCGGTTAA

3451 GGCTAAACTG AAAGGACAAG TTTTGGTGAC TGCGCTCCTC CAAGCCAGTT

3501 ACCTCGGTTC AAAGAGTTGG TAGCTCAGAG AACCTTCGAA AAACCGCCCT

3551 GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGCG CAGACCAAAA

3601 CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTC

3651 AGTGCAATTT ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT

3701 ATAAGTTGTT AATTCTCATG TTTGACAGCT TATCATCGAT

Fig. 3a

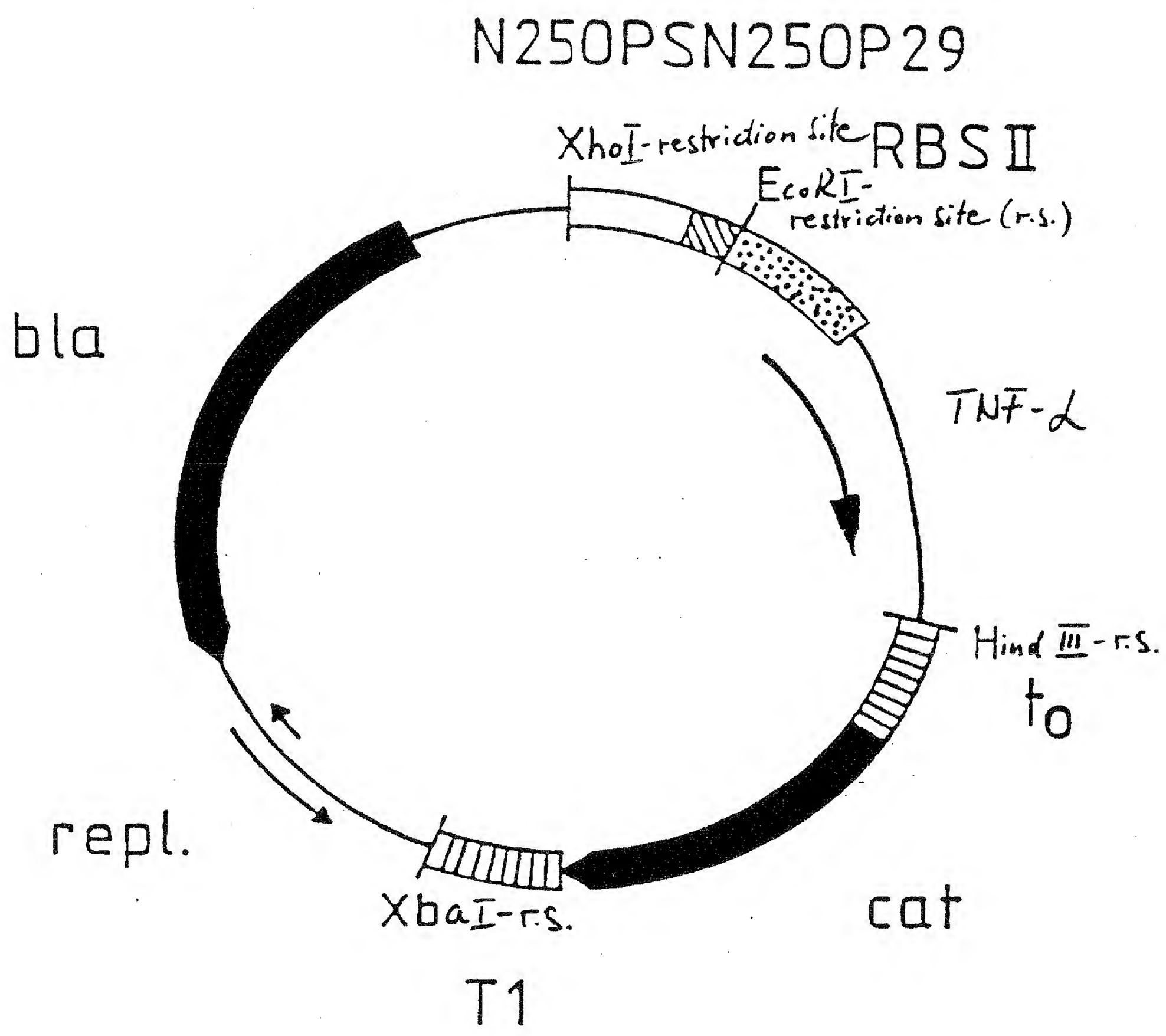


Fig. 3b/1

XhoI

```
   1  CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
                                                EcoRI
  51  AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
 101  AGGAGAAATT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
 151  CCTGTAGCCC ATGTTGTCGC GAACCCTCAA GCTGAGGGGC AGCTCCAGTG
 201  GCTGAACCGC CGGGCCAATG CCCTCCTGGC CAATGGCGTG GAGCTGAGAG
 251  ATAACCAGCT GGTGGTGCCA TCAGAGGGCC TGTACCTCAT CTACTCCCAG
 301  GTCCTCTTCA AGGGCCAAGG CTGCCCCTCC ACCCATGTGC TCCTCACCCA
 351  CACCATCAGC CGCATCGCCG TCTCCTACCA GACCAAGGTC AACCTCCTCT
 401  CTGCCATCAA GAGCCCCTGC CAGAGGGAGA CCCCAGAGGG GGCTGAGGCC
 451  AAGCCCTGGT ATGAGCCCAT CTATCTGGGA GGGGTCTTCC AGCTGGAGAA
 501  GGGTGACCGA CTCAGCGCTG AGATCAATCG GCCCGACTAT CTCGACTTTG
 551  CCGAGTCTGG GCAGGTCTAC TTTGGGATCA TTGCCCTGTG AGGAGGACGA
 601  ACATCCAACC TTCCCAAACG CCTCCCCTGC CCCAATCCCT TTATTACCCC
 651  CTCCTTCAGA CACCCTCAAC CTCTTCTGGC TCAAAAAGAG AATTGGGGGC
                HindIII
 701  TTAGGGTCGG AACCCAAGCT TGGACTCCTG TTGATAGATC CAGTAATGAC
 751  CTCAGAACTC CATCTGGATT TGTTCAGAAC GCTCGGTTGC CGCCGGGCGT
 801  TTTTTATTGG TGAGAATCCA AGCTAGCTTG GCGAGATTTT CAGGAGCTAA
 851  GGAAGCTAAA ATGGAGAAAA AATCACTGG ATATACCACC GTTGATATAT
 901  CCCAATGGCA TCGTAAAGAA CATTTTGAGG CATTTCAGTC AGTTGCTCAA
 951  TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC
1001  CGTAAAGAAA AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG
1051  CCCGCCTGAT GAATGCTCAT CCGGAATTTC GTATGGCAAT GAAAGACGGT
1101  GAGCTGGTGA TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA
1151  GCAAACTGAA ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC
1201  GGCAGTTTCT ACACATATAT TCGCAAGATG TGGCGTGTTA CGGTGAAAAC
1251  CTGGCCTATT TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC
```

Fig. 3b/2

```
1301  CAATCCCTGG GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG

1351  ACAACTTCTT CGCCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC
1401  GACAAGGTGC TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTCTGTGA

1451  TGGCTTCCAT GTCGGCAGAA TGCTTAATGA ATTACAACAG TACTGCGATG

1501  AGTGGCAGGG CGGGGCGTAA TTTTTTTAAG GCAGTTATTG GTGCCCTTAA

1551  ACGCCTGGGG TAATGACTCT CTAGCTTGAG GCATCAAATA AAACGAAAGG

1601  CTCAGTCGAA AGACTGGGCC TTTCGTTTTA TCTGTTGTTT GTCGGTGAAC

                                  XbaI
1651  GCTCTCCTGA GTAGGACAAA TCCGCCGCTC TAGAGCTGCC TCGCGCGTTT

1701  CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA

1751  CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG

1801  TCAGCGGGTG TTGGCGGGTG TCGGGGCGCA GCCATGACCC AGTCACGTAG

1851  CGATAGCGGA GTGTATACTG GCTTAACTAT GCGGCATCAG AGCAGATTGT

1901  ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA TGCGTAAGGA

1951  GAAAATACCG CATCAGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG

2001  CGCTCGGTCT GTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT

2051  AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG

2101  CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG

2151  TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC

2201  AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC

2251  CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC

2301  GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG

2351  CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG

2401  GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT

2451  AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC

2501  AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA

2551  CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA

2601  TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG

2651  TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG
```

Fig. 3b/3

2701 TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT

2751 TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA

2801 AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT

2851 TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT

2901 TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT

2951 CTGTCTATTT CGTTCATCCA TAGCTGCCTG ACTCCCCGTC GTGTAGATAA

3001 CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG

3051 CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC

3101 CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC

3151 AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT

3201 AGTTTGCGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC

3251 GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG

3301 TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT

3351 CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT

3401 GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT

3451 CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG

3501 CGACCGAGTT GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA

3551 TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA

3601 AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT

3651 CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG

3701 GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA

3751 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA TTATTGAAGC

3801 ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA

3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC

3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG

3951 CGTATCACGA GGCCCTTTCG TCTTCAC

Fig. 4

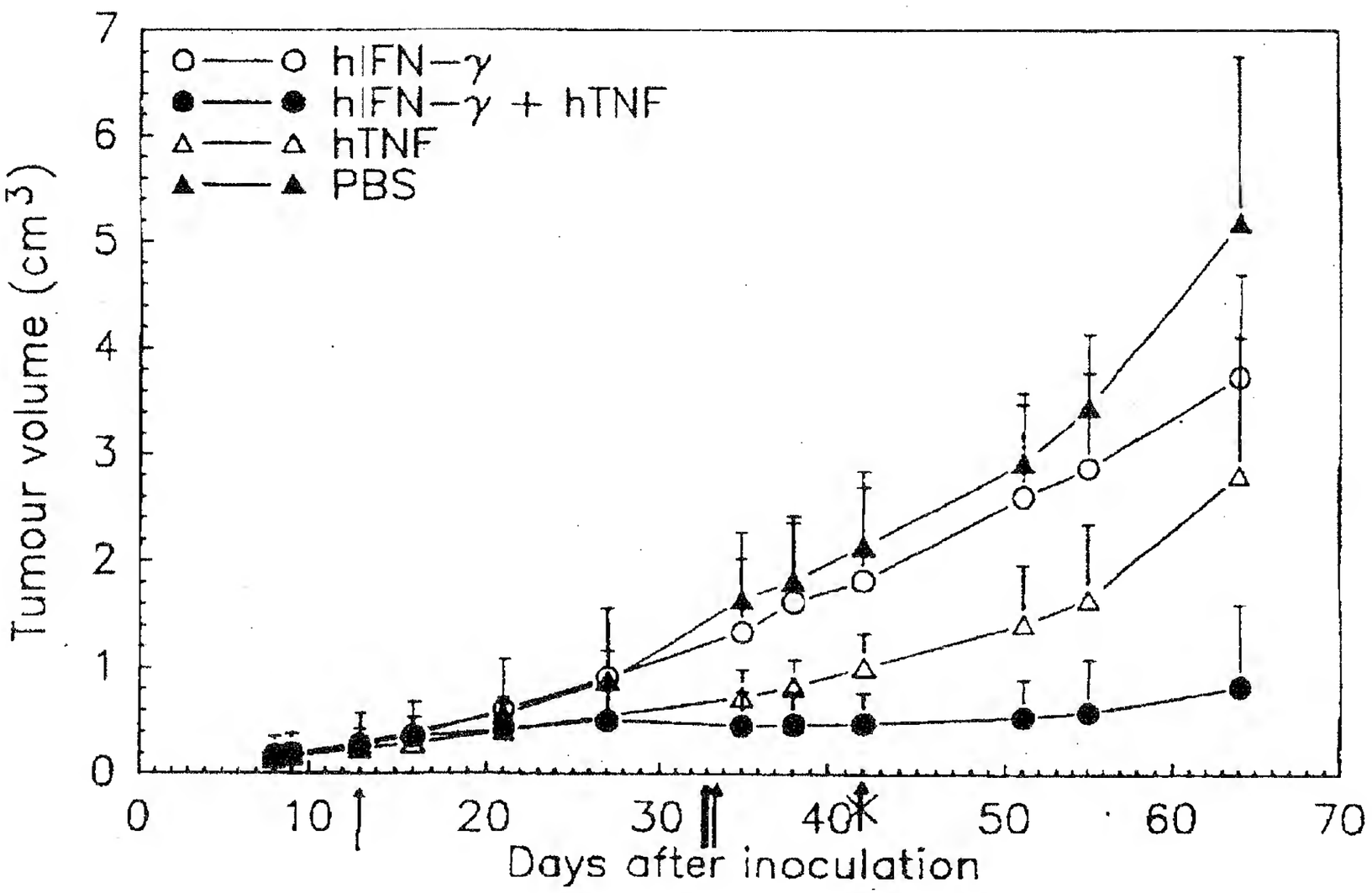


Fig. 5

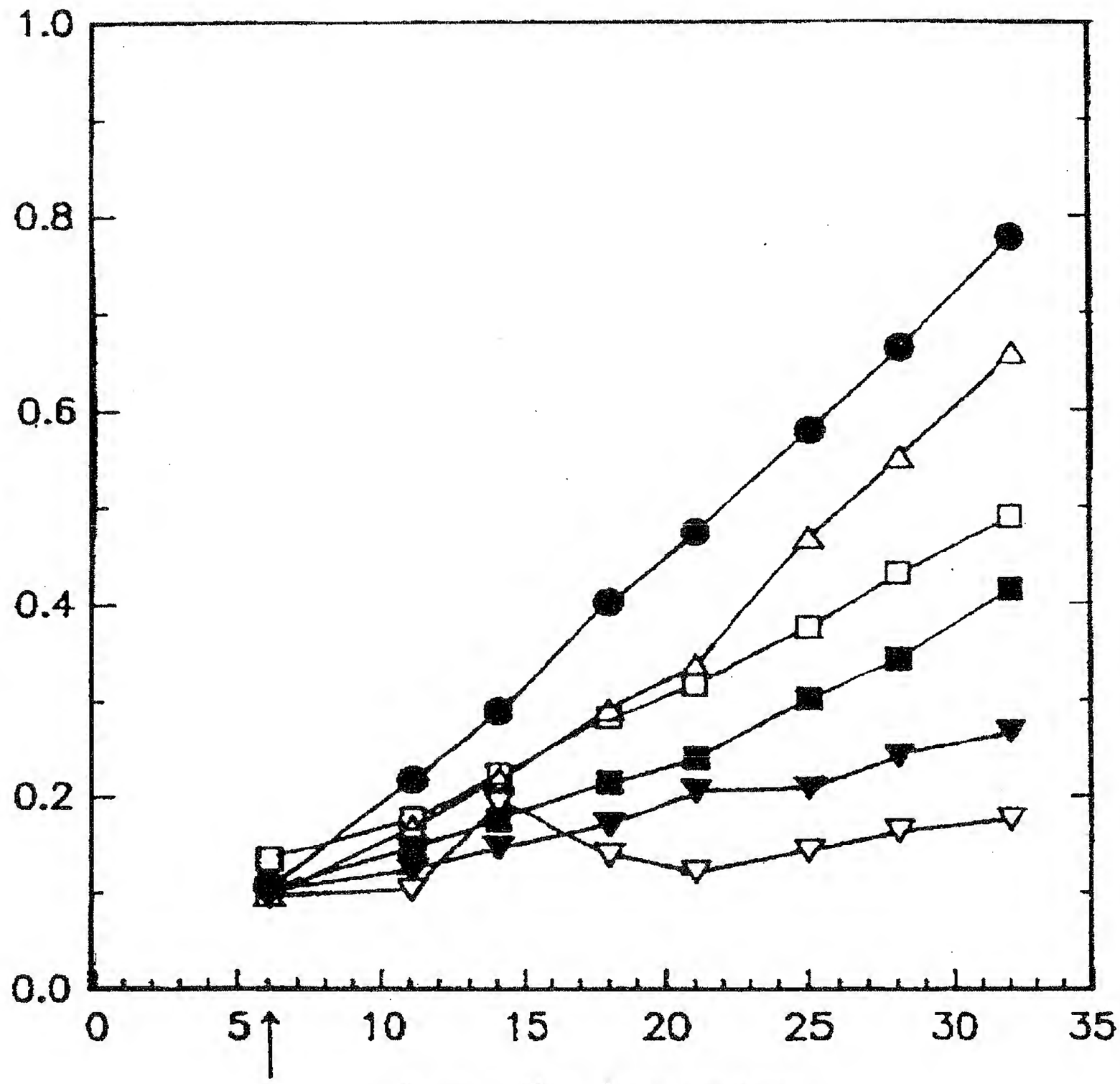
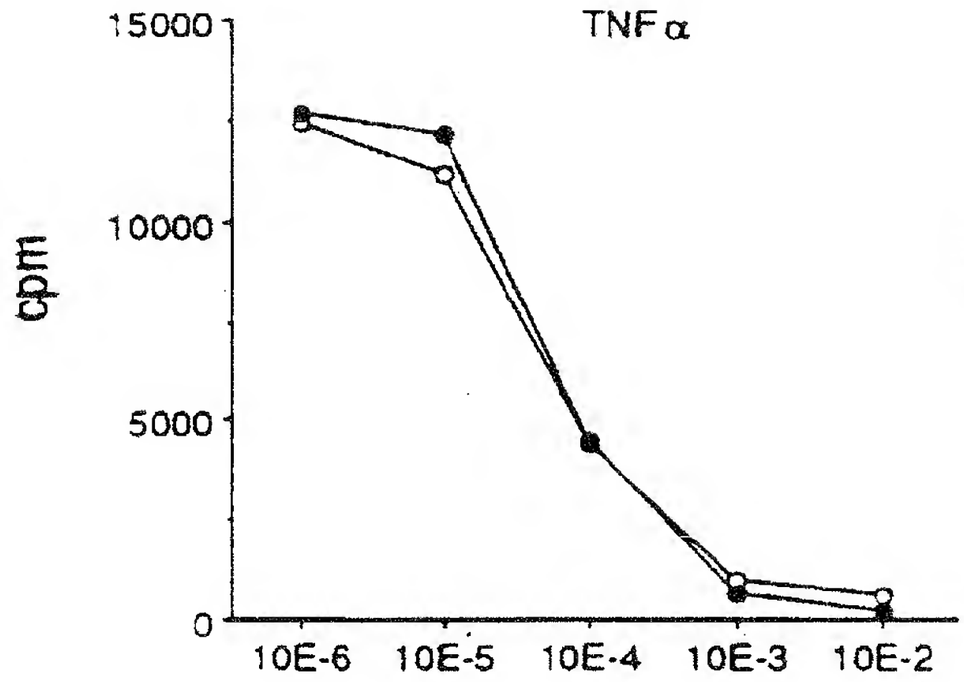
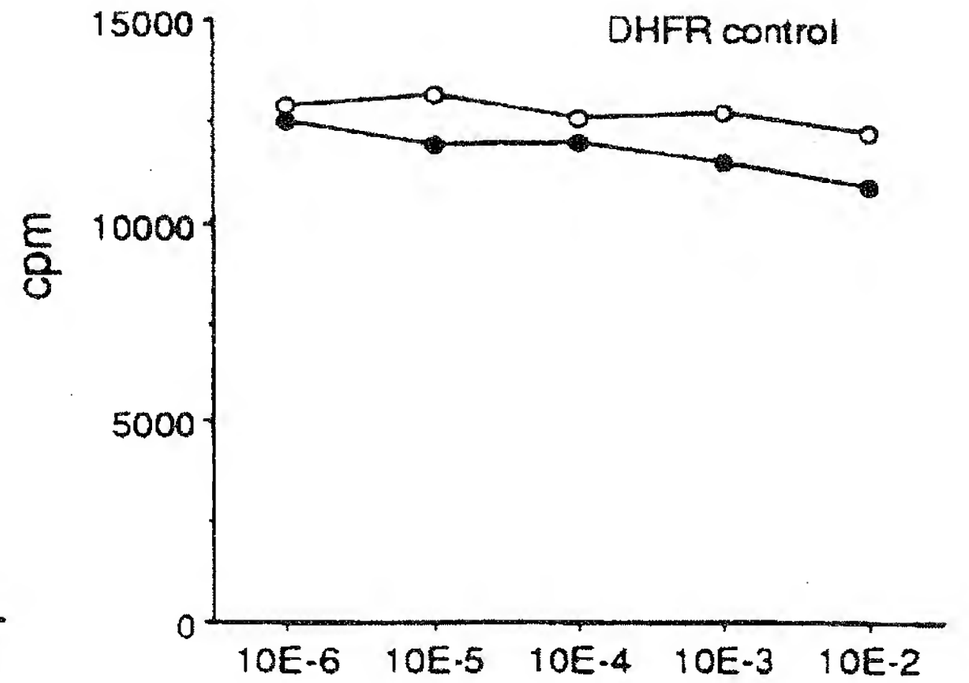
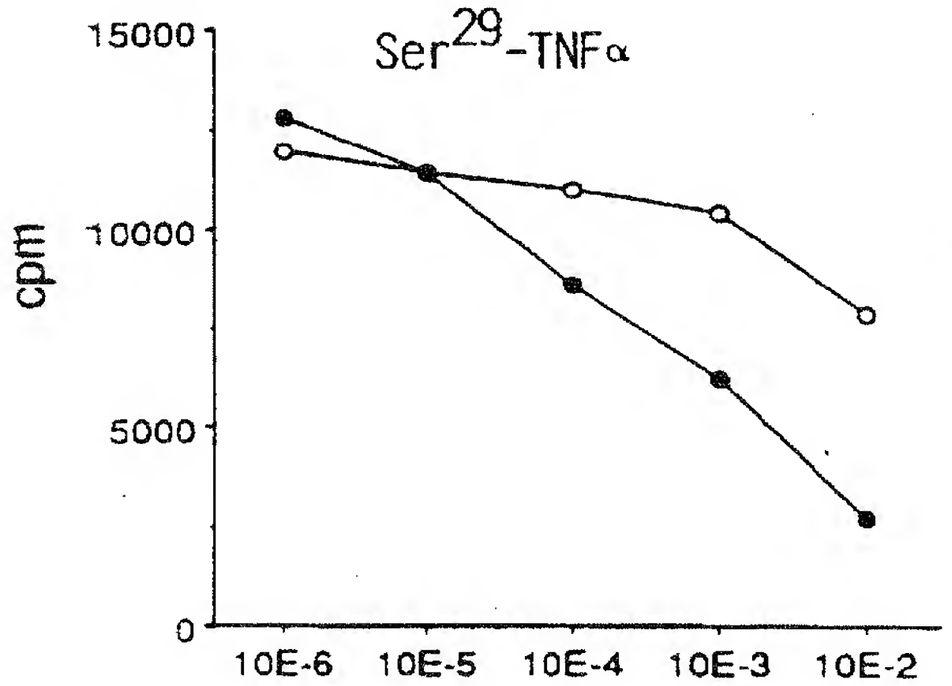
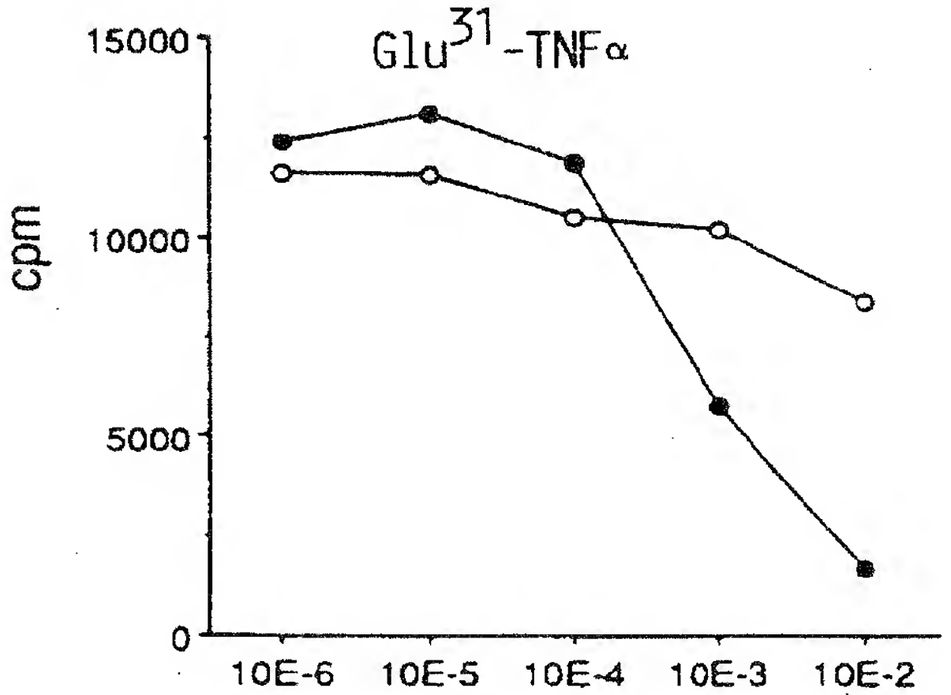


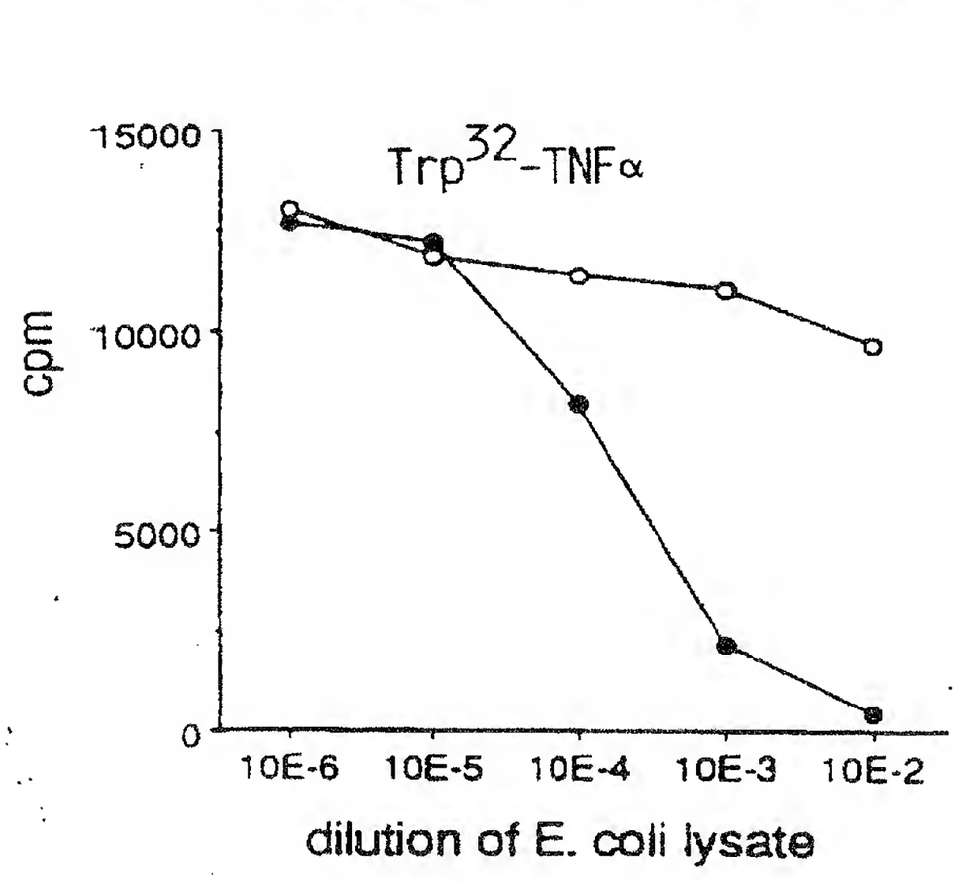
Fig. 6











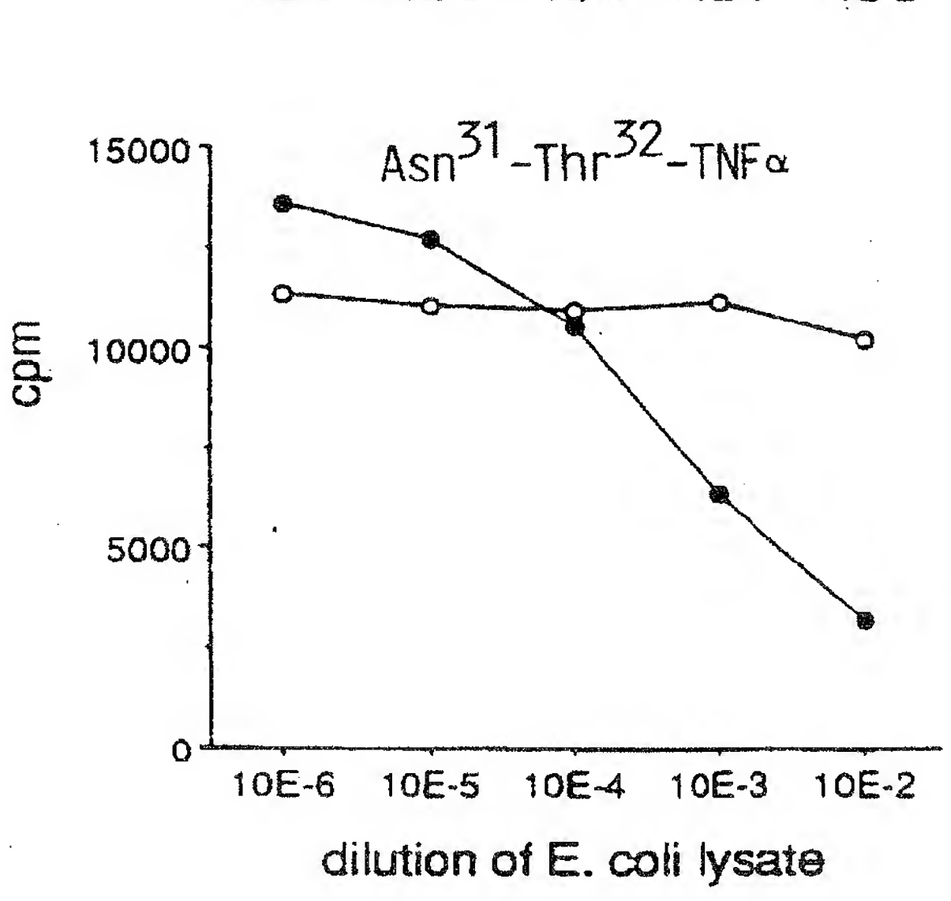


Fig. 7

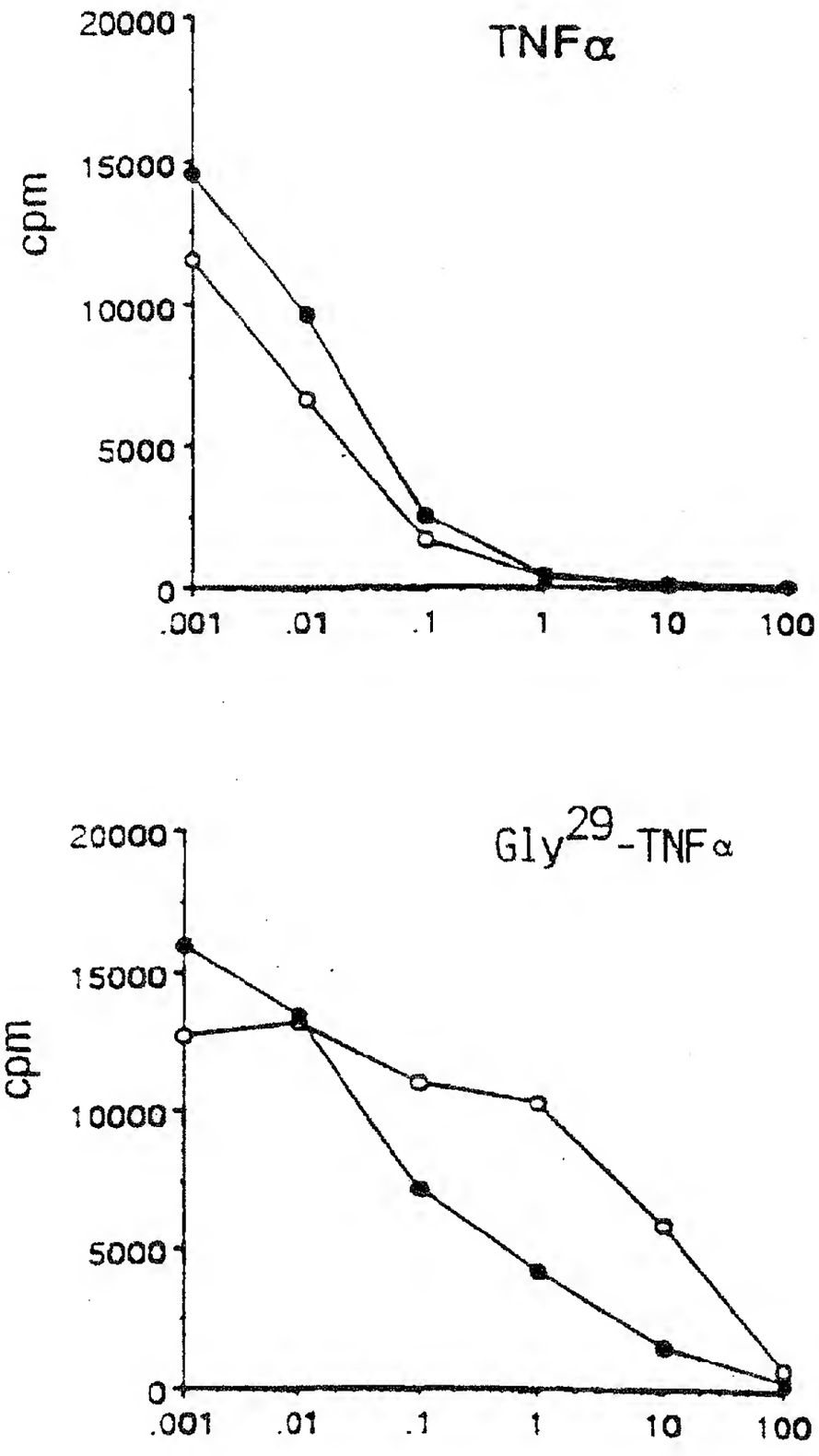
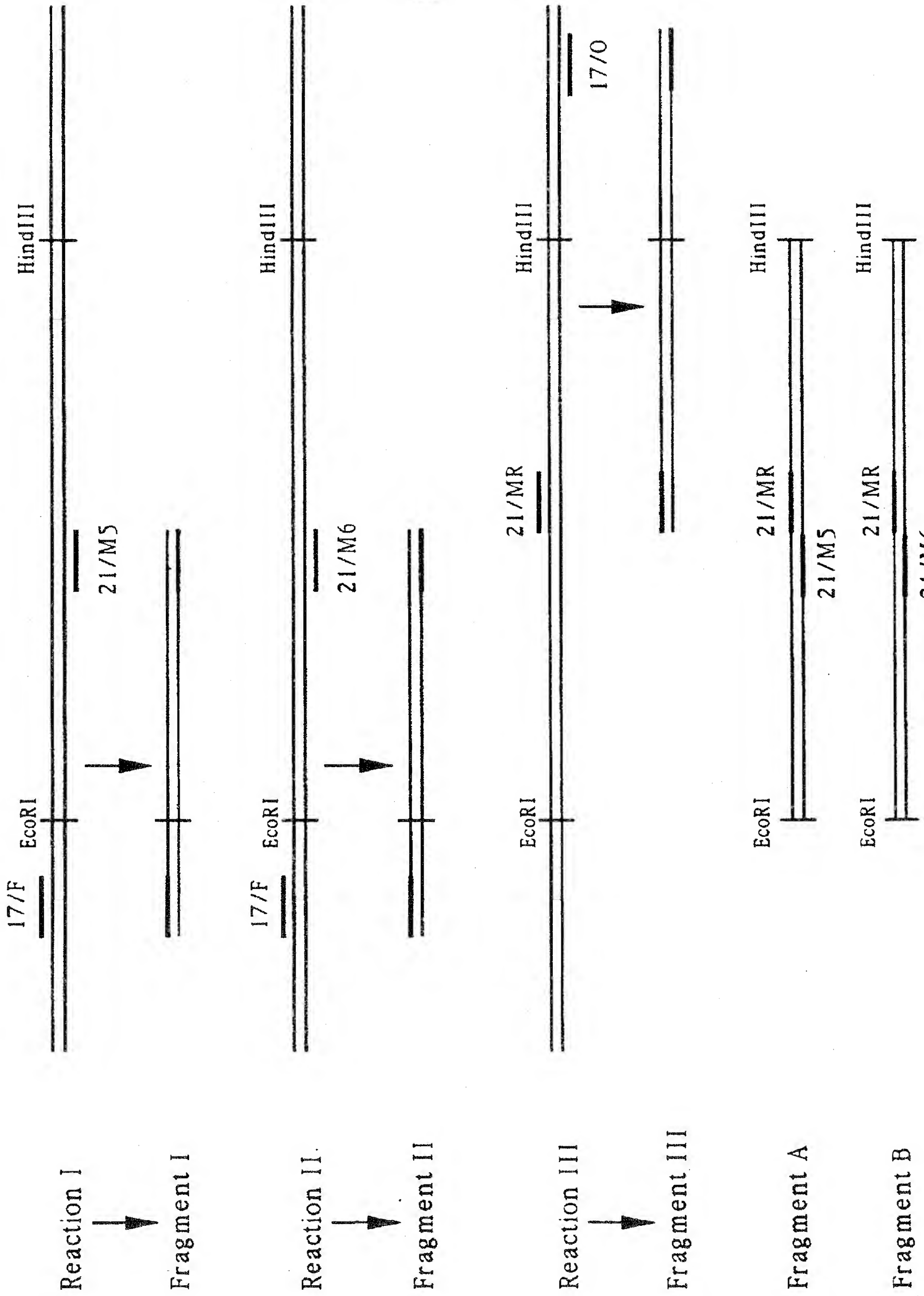


Fig. 8



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(71) Applicant: **F. HOFFMANN-LA ROCHE AG**
Postfach 3255
CH-4002 Basel(CH)

(72) Inventor: **Fiers, Walter**
3 Beukendreef
B-9070 Destelbergen(BE)
Inventor: **Tavernier, Jan**
2 Bottelweg
B-9860 Balegem(BE)
Inventor: **Van Ostade, Xaveer**
46 Kipdorpvest
B-2000 Antwerp(BE)

(74) Representative: **Mezger, Wolfgang, Dr. et al**
Grenzacherstrasse 124 Postfach 3255
CH-4002 Basel(CH)

(54) TNF-Muteins.

(57) It is an object of the present invention to provide a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor, DNA sequences coding for such muteins, vectors comprising such DNA sequences, host cells transformed with such vectors and a process for the production of such muteins employing such transformed host cells and pharmaceutical compositions containing such muteins and their use for the treatment of illnesses, e.g. cancer.

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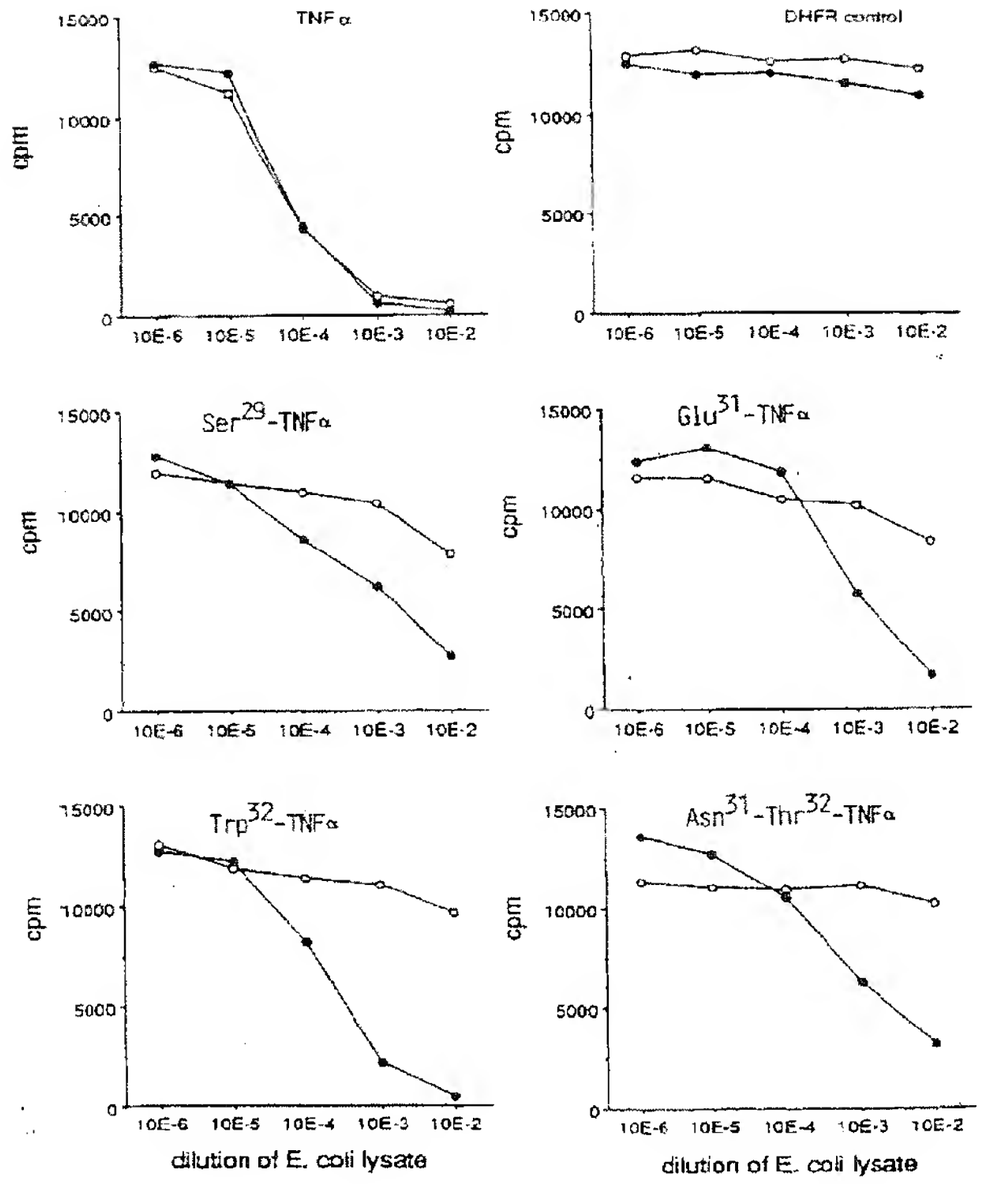
Fig. 6



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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number

EP 91 11 9128

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	JOURNAL OF BIOCHEMISTRY, vol. 101, 1987, pages 919-925, Japanese Biochemical Society, Tokyo, JP; M. TSUJIMOTO et al.: "Comparative studies of the biological activities of human tumor necrosis factor and its derivatives" * Whole article, especially figure 1 * ---	1-3,15-16,18-25	C 12 N 15/28 C 12 P 21/02 C 07 K 13/00 C 12 N 1/21 // A 61 K 37/02 (C 12 N 1/21 C 12 R 1:19)
D,X	WO-A-8 806 625 (CETUS CORP.) * Page 17, lines 16-33; page 14, lines 15-31; claims * ---	1-3,8,11-12,15-25	
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TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N
C 07 K
C 12 P

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Remark: Although claim 25 is directed to a method of treatment of the human body (Art. 52 (4) EPC) the search has been carried out and based on the alleged effects of the compound.

Place of search	Date of completion of the search	Examiner
THE HAGUE	28-07-1992	LE CORNEC N.D.R.

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Application Number

EP 91 11 9128

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	PROTEIN ENGINEERING INTERNATIONAL CONFERENCE CENTER, Kobe, 20th - 25th August 1989, pages 375-376, Eynsham, Oxford, GB; T. MASEGI et al.: "Fundamental studies on novel recombinant tumor necrosis factor mutants" * Whole document * ---	1-3,8, 17-21	
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X	PATENT ABSTRACTS OF JAPAN, vol. 13, no. 119 (C-579)[3467], 23rd March 1989; & JP-A-63 291 590 (TEIJIN LTD) 29-11-1988 * Whole abstract * ---	1-3,8, 18-25	
P,X	THE EMBO JOURNAL, vol. 10, no. 4, April 1991, pages 827-836, Eynsham, Oxford, GB; X. VAN OSTADE et al.: "Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis" * Whole article * -----	1-5,8-9 ,18-25	
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EPO FORM 1503 03.82 (P04C10)